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Abstract

The pathogenicity of spore-forming bacteria such as *Bacillus cereus* has become a global food safety concern. Unlike sterilization, bacillus spores can survive pasteurization temperatures. Conventional thermal treatments remain the major processing technique in many food industries but due to their drawbacks in reducing the taste and nutritional qualities of food, the use of non-thermal technologies such as High-Pressure Processing (HPP) has increased in recent years. The main objective of this study was to investigate the effect of thermal and pressure-thermal treatments in inhibiting *B. cereus* NVH 1230-88 spore and vegetative cell growth in broth and food matrices. The effect of initial bacterial numbers on the growth of *B. cereus* NVH vegetative cells was investigated. Higher cell numbers resulted in a faster time to detection (TTD) at OD of 0.2. The effect of preservatives (NaCl, KCl, $NaNO_2$ and nisin) at different concentrations and pH on *B. cereus* NVH 1230-88 spore growth was studied. The combined effect of the preservatives on bacterial growth was also investigated. No time to detection (TTD) values were recorded at high salt concentrations of 4% and 2% (NaCl and KCl) at pH 4, illustrating inhibitory effect of high concentrations of preservatives at lower pH. $NaNO_2$ exhibited no inhibitory effect on the growth of *B. cereus* NVH 1230-88 spores. Inactivation of spores by thermal treatments at 97℃ for 5 and 10 mins in LB medium resulted in a log reduction of 3.74 and 4.10 respectively. However, under the same processing conditions, a log reduction of 3.35 log cfu/ml was achieved in minced meat. The effect of spore inactivation by HPP (600mPa) at three different processing temperatures (20, 40 and 55℃) on the *B. cereus* NVH spores resulted in log reductions of 0.22 cfu/ml, 1.80 cfu/ml, and 3.08 cfu/ml respectively, illustrating the synergistic effect of pressure thermal treatments in spore inactivation. HPP alone could not achieve the highest spore inactivation in minced meat. However, a synergy of pressure-thermal (HPP-55℃) treatments resulted in an increase in spore reduction in the meat matrix. High spore inactivation and log reductions were observed in meat samples treated with 1% NaCl, 500IU nisin at HPP 55℃. A similar trend was observed when a similar experiment was performed in Bioscreen. The vegetative cell growth of *B. cereus* NVH 1230-88 was tested at 8℃ and 15℃ with a combination of preservatives (NaCl adjusted at pH 4). Higher bacterial log numbers were recorded at 15℃ compared to 8℃ in samples with no preservatives. Bacterial log numbers declined in samples with preservatives. However, temperature and time played an important role in the extent of inhibition caused by the preservatives.

Abbreviations

a_w	Water activity
CFU	Colony forming units
D-value	Decimal reduction time
DPA	Dipicolinic acid
HPP	High pressure processing
n.g	No growth
Milli-Q water	Pure water
MPa	Megapascal
OD	Optical density
PATS	Pressure-assisted thermal sterilization
rpm	Rotation per minute
TTD	Time to detection

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Acknowledgement

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1. Introduction

The rise in food borne illnesses has necessitated stringent quality and safety checks along the food production chain. However, the underlying cause of these reported cases still stands. The persistence of foodborne pathogens in food products, niches and food production environments have contributed to increased morbidity and mortality, posing a global food safety concern. In recent years, the consumers quest to maintain the freshness and nutritional value of foods have led to the emergence of minimally processed and ready-to-eat (RTE) food which have been largely attributed to the increase in food related illnesses. Reports from the Center for Disease Control and Prevention (CDC) showed that 9.4 million episodes of foodborne illness, resulting in 55,961 hospitalizations and 1,351 deaths were caused by major known pathogens each year (Scallan et al., 2011). Research by the WHO (2003) also estimated that foodborne diseases may result in 76 million illnesses, 325,000 hospitalizations and 5000 deaths each year in the U.S (Organization, 2003; Scharff, 2012). According to EU data, bacterial toxins from *Bacillus cereus*, *Clostridium*, and *Staphylococcus* accounted for 17.7% (2016) and 15.9% (2017) of all registered food- and waterborne outbreaks, trailing only *Salmonella* (European Food safety Report, 2018). *Bacillus cereus* is suspected of causing 1.4% to 12% of all food poisoning outbreaks worldwide (Grutsch et al., 2018). Out of the 98 registered outbreaks in the EU in 2018, *B. cereus* toxins ranked fifth, trailing *Salmonella*, *Campylobacter*, *norovirus*, and *Staphylococcus* toxins.

Bacillus cereus enterotoxins in food matrices have become a major food safety concern because thermophilic endospores produced by *Bacillus cereus* can resist pasteurization processes and can be revived by germination, outgrowth, and production of spoilage enzymes (proteases, lipases, and phospholipases) that are harmful to consumer health (Lücking et al., 2013). *B. cereus* growth and survival under refrigeration temperatures is also a major threat in food processing and is considered a microbiological hazard. According to European Food Safety Authority guidelines, *B. cereus* growth temperatures typically range from 4°C to 55°C (Authority, 2005; Setlow, 2006).

B. cereus is considered to be a common contaminant of raw milk and rice (Haque et al., 2022; Tewari & Abdullah, 2015). Research studies have also reported the prevalence of *B. cereus* in infant foods which is of a major concern. According to studies by (Rahimi et al., 2013), 42 % samples of infant foods, positive for the presences of *B. cereus* and its enterotoxigenic genes were reported. The results indicated 30 to 93 *B. cereus* spores per gram sample. Research by

(Reyes et al., 2007) have also documented the presence of *B. cereus* in dried milk products. They showed 45.9 % incidence of *B. cereus* in dried milk products (milk with rice, milk substitute, milk powder, milk-cereal-rice, pudding milk, flan, and mousse) which were used by the Chilean School Feeding Program. (Chitov et al., 2008) found all pasteurized milk samples positive for *B. cereus* and the bacterial count varied between 50 and 1.7×10^3 cfu/g. This confirmed the ability of *B. cereus* spores to survive pasteurisation temperatures and recontamination by processing equipments and surfaces as documented by (Kumari & Sarkar, 2016; Silva et al., 2018). Psychrotrophic *B. cereus* group bacteria have also been associated with contaminating meat and meat products (Webb et al., 2019; Wijnands et al., 2006). It can also compromise the microbiological quality of eggs and their products (Moschonas et al., 2021; Techer et al., 2020). *B. cereus* has also been isolated from spices, chocolates, cereals, and cereal derivatives (Rahnama et al., 2022; Tewari & Abdullah, 2015).

In an attempt to prevent the contamination of *B. cereus* in food matrices, many processing techniques have been applied to avoid the possible survival of *Bacillus cereus* spores in foods. Notably, thermal processing such as pasteurization and mild heat treatments ranging between 65°C and 95°C have been explored but these temperatures are inadequate to kill bacterial spores (Oomes et al., 2009). According to research studies, these heat treatments may kill the vegetative cells but may create favourable conditions which may activate dormant spores. The activation of dormant spores by sublethal heating induces germination thus the conversion of spores to vegetative cells (Løvdal et al., 2011; Xing & Harper Jr, 2020). Modified Tyndallization techniques such as double heat treatments at specifies temperatures have also proved promising but due to the effect of thermal treatments on the sensory, nutritional and organoleptic properties of food, more attention has been tailored towards non-thermal processing methods (Cullen et al., 2012; Hassoun et al., 2020). High pressure processing (HPP) is a novel technique that has gained acceptance in the food industry in the past decade (Bi et al., 2020). Research studies have revealed the potential benefits of high-pressure processing as an alternative to conventional heat treatments in inactivating microorganisms in food and retaining the nutritional quality and apparent freshness of the final product (Silva, 2019). Although HPP presents a possible solution to producing valued-added foods of superior quality and shelf-life, there is the need to fundamentally understand the dynamics of reducing of bacterial spores by pressure and the potential of coupling HPP and other hurdle technologies to inactivate bacterial spores in food matrices.

Aim of the study

The aim of this study was to investigate the temperature tolerance of spores and the inhibition of bacteria growth by preservatives. The effect of thermal and pressure-thermal treatments in inhibiting *B. cereus* NVH spore and vegetative cell growth in broth and food matrices was studied. The efficacy of combined preservatives in *B. cereus* NVH inactivation was also investigated. This knowledge may be helpful in controlling *B. cereus* in foods with mild processing techniques. The flow diagram (Figure 1) gives an overview of all experiments carried out in this study.

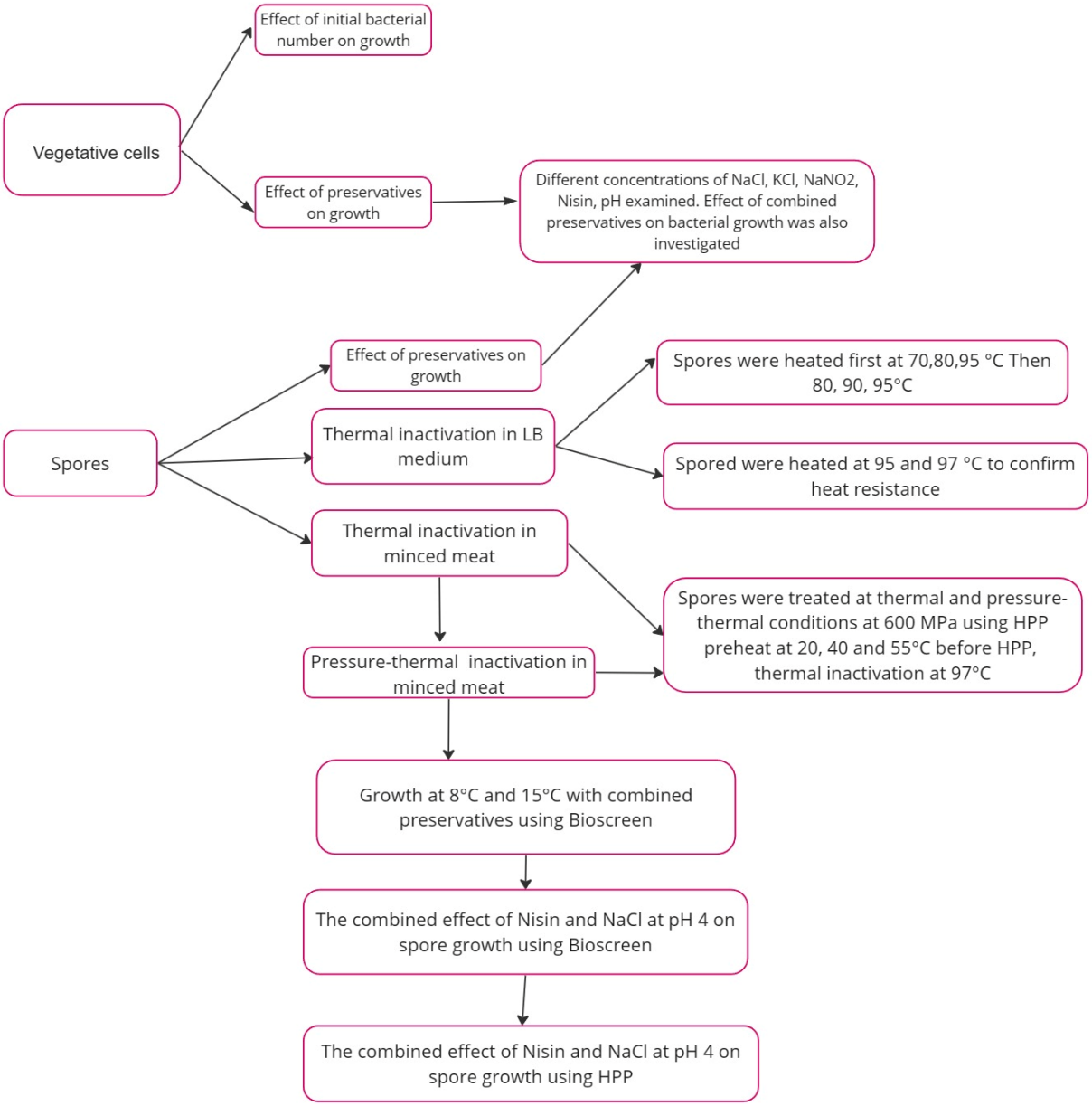


Figure 1: Flow diagram of experiments used in this study

2. Theory

2.1. The genus *Bacillus*

Bacteria of the genus *Bacillus* are widely distributed in nature. They are aerobic, endospore-forming, gram-positive rods, which include thermophilic, psychrophilic, acidophilic, alkalophilic, and halophilic bacteria (Harwood, 1989; Nazina et al., 2001). The *Bacillus* genus was discovered in 1872 by Ferdinand Cohn, who changed the name of Ehrenberg's (1835) "*Vibrio subtilis"* to "*Bacillus subtilis"*(Harwood, 1989). Members of this genus are one of the most diverse bacterial genera, widely distributed in soil, air and water (Te Giffel et al., 1996). They possess a wide range of commercially beneficial properties, with some species (*B. macerans and B. polymyxa*) known to promote nitrogen fixation (Ding et al., 2005). The tolerance of certain strains to high and low temperatures and pH ranges have ascribed some industrial importance to these strains as commercial sources of enzymes with fermentative abilities (Norris et al., 1981).

2.1.1 Properties of *B. cereus*

Bacillus species exist in both terrestrial and aquatic conditions, such as soils, dusts, and water. They are known to also exist in the gut of various insects and animals, and are a common soil saprophyte (Hong et al., 2009). Members of this genus are facultative, aerobic spore-forming, Gram-positive and rod-shaped bacteria growing singly, in pairs, chains or as long filaments. They include both motile and non-motile species. Their cell length range between 0.9-10 μm and the diameter from 0.4-1.8 μm. Most of the species are mesophilic with minimum growth temperature 5-20℃, maximum 35-55℃ and optimum about 30℃. Some representatives of this genus are thermophilic, psychrophile, acidophilic or alkaliphilic and salt tolerant to halophilic (Løvdal et al., 2011) (Kathariou & Oyarzabal, 2014). From the family *Bacillaceae*, the genus *Bacillus* is subdivided into groups namely the *B. cereus* group and *B. subtilis* group, despite the difficulties in classification. The *B. subtilis* group include *B. pumilus, B. licheniformis, B. amyloliquefaciens, B. atrophaeus, B. sonorensis, B. subtilis, B. vallismortis and B. mojavensis.* Members of this group are mesophilic and neutrophilic bacterial species (Løvdal et al., 2011). According to (Granum & Lindbäck, 2012), the *B. subtilis* group of species can produce toxins associated with food poisoning, which is detrimental to the health of consumers. The *B. cereus* group consists of *B. cereus, B. anthracis, B. mycoides, B. megaterium, B. weihenstephanensis and B. thuringiensis*. Most are mesophilic but some are psychrotolerant. This group is also known for its pathogenic properties, causing foodborne illness (Løvdal et al., 2011). Due to taxonomic challenges, several methods and criteria for the identification and classification of

members of the *Bacillus* genus have emerged. These include identification based on chemotaxonomic characteristics, genomic characteristics by the 16S rRNA gene sequence analysis, traditional biochemical tests, morphological and physiological characteristics (Løvdal et al., 2011; Sacchi et al., 2002). One characteristic property of the *Bacillus* species is the formation of endospores in response to adverse growth conditions. At sublethal heat treatments, dormant endospores may be activated to germinate into vegetative cells, which is of a lower heat resistance than the dormant spores (Løvdal et al., 2011).The presence of *Bacillus* spores in food matrices is a food safety concern because these spores may produce toxins which can lead to food poisoning when ingested by consumers (Stenfors Arnesen et al., 2008).

2.1.2. *Bacillus cereus*

Bacillus cereus is a rod-shaped aerobic or facultative anaerobic Gram-positive, motile, spore forming bacterium commonly found in the environment or as a contaminant of foods. It is a ubiquitous saprophyte which mostly inhabit soil, water, air and vegetables. In the soil, it can be found at concentrations of about 10^6 cfu/ml (Hendriksen et al., 2006). Generally, *B. cereus* is resistant to low humidity, high temperatures, dehydration, radiation, and acidity but these properties are strain dependent. *Bacillus cereus* strains can grow in a moderately wide range of temperatures. Most variants can be considered mesophilic (optimal growth temperature of 37℃ and survival below 10 ℃). However, psychrotropic strains exist with optimal growth temperatures below 10℃ (Afchain et al., 2008). Research studies have shown that the *B. cereus* can grow at temperatures ranging from 4℃ to 55 °C but 30 ℃ to 40 °C are optimal, depending on the strain (Bintsis, 2017). However, research studies have also documented the limited growth of this bacteria in meat at pH 4.3 (Bintsis, 2017). Some strains are quite resistant to salting but are easily inactivated by thermal treatments such as pasteurization, Tyndallization or cooking at temperatures above 90℃ (Majed et al., 2016).

B. cereus has been linked to a variety of complex foods, including rice, grains, dairy products, spices, dried foods, and vegetables. Nonetheless, other food products, such as mildly processed foods and fresh foods, have been a source of contamination and have been implicated in *B. cereus* intoxication (Kim et al., 2010). Due to the adhesive properties of *B. cereus* spores, foods can become contaminated during processing when they circulate through pipes, work surfaces, or belts (Jullien et al., 2003). Spores of *B. cereus* have also been isolated in ready-to-eat foods since the vegetative cells are mostly destroyed by thermal processes. Food processes that allow *B. cereus* spores to germinate and may represent a risk to consumers include storage of processed products without refrigeration or under temperature abuse, or the use of raw

materials in complex foods (Hong et al., 2008). Research carried out on raw rice show that *B. cereus* spores are frequently isolated from this food, due to its ubiquity in nature. In fact, a prevalence of 100% was observed in 2010 in Argentina (Fangio et al., 2010). Similarly, in Colombia, 244 samples of rice-containing foods were tested in various regions of the country, and the results revealed that 11.92% of those foods had concentrations higher than 10^4 cfu/g, which is considered high risk. *B. cereus* has been the most common cause of food poisoning outbreaks in Europe in recent years, particularly in the Netherlands and Hungary (Haque et al., 2021; Munshi et al., 2021). Cases of *B. cereus* food poisoning have also been reported in the United States, the United Kingdom, and Norway (Tewari & Abdullah, 2015)

2.1.3. *Bacillus cereus* Toxins

B. cereus is known to produce two different types of illnesses, the emetic type and diarrhoeal type, depending on the context at which it grows (Rodrigo et al., 2021). The diarrhoeal type is produced when a large number of vegetative cells or spores are ingested and the bacterium passes the stomach barrier, during their growth in the small intestine (Fagerlund et al., 2007). The diarrhoeal type is caused by enterotoxins (Hbl, Nhe or CytK) produced by *B. cereus* cells growing in the hosts small intestine. It has an incubation period of 8 - 16 h (occasionally > 24 h), with characteristic symptoms such as abdominal pain, watery (occasionally bloody) diarrhoea and sometimes nausea (Løvdal et al., 2011). Generally, the symptoms disappear after 12 - 24 h, but can less frequently remain after several days. Foods most frequently implicated are meat products, soups, vegetables, pudding/sauces, and milk/milk products (Granum & Lund, 1997; Kramer & Gilbert, 1989; Stenfors Arnesen et al., 2008). The infective dose of diarrhoeal *B. cereus* is assumed to be in the range $10^5 – 10^7$, and the lowest dose is probably associated with ingestion of spores that are resistant to the stomach acid (Granum & Lindbäck, 2012). Unlike the diarrhoeal type, the emetic toxin is characterised by cyclic cereulide peptide, produced during the growth of *B. cereus* in the food before consumption, when the conditions of pH, water activity, and temperature are favourable. Due to its strong hydrophobic character, it must be attached to or dissolved in a food matrix to cause food poisoning (Rodrigo et al., 2021).The toxin is resistant to heat, pH and proteolysis and hence, can withstand cooking and passage through the stomach (Granum & Lindbäck, 2012). It has a shorter incubation period of 0.5 - 5h, with subsequent symptoms such as nausea, vomiting and malaise, occasionally followed by diarrhoea. The symptoms generally resolve after 12 - 24 hrs. A concentration of $10^5 – 10^8$ *B. cereus* cells/g is probably needed to produce enough emetic toxin to induce food poisoning (Granum & Lindbäck, 2012).

A comparison of the characteristics of the Emetic and Diarrhoeal-type illnesses are made in *Table 1.*

Table 1: Characteristics of the two types of B. cereus foodborne diseases (Stenfors Arnesen et al., 2008).

Characteristics	Diarrhoeal disease	Emetic disease
Type of toxin	Protein; enterotoxin(s): Hbl, Nhe, CytK	Cyclic peptide; emetic toxin (cereulide)
Location of toxin production	In the small intestine of the host	Preformed in foods
Symptoms	Abdominal pain, watery diarrhoea and occasionally nausea, Lethality has occurred	Nausea, vomiting and malaise. A few lethal cases (possibly due to liver damage)
Incubation time	8–16 h (occasionally >24 h)	0.5–6 h
Duration of illness	12–24 h (occasionally several days)	6–24 h
Foods most frequently implicated	Proteinaceous foods; meat products, soups, vegetables, puddings, sauces, milk and milk products	Starch-rich foods; Fried and cooked rice, pasta, pastry and noodles

2.1.4. Vegetative cell growth

Two distinct states of *B. cereus* species exist, the vegetative and spore state. In the vegetative state, bacteria are metabolically active and use available nutrients to grow and replicate. Vegetative cells divide by binary fission, a process that generates two identical daughter cells from a single mother cell (Angert, 2005). A bacterial growth curve depicts the pattern of growth of a bacterial population in a growth medium. This curve is depicted as the number of live cells in a population over time and consists of four phases: lag, exponential (logarithmic), stationary, and death (decline) (Schaechter, 2015).

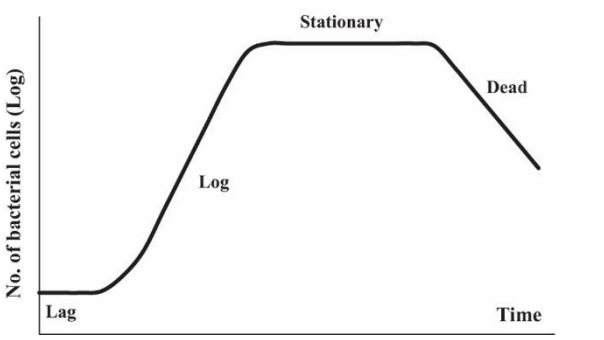


Figure 2: Graphical representation of typical bacterial growth curve in culture medium. Stages of bacterial growth include the lag, log, stationary and dead phases. Source:(Wang et al., 2015).

The lag phase is characterized by the adjustment of the bacteria to new environments. In the lag phase the bacteria are involved in various cellular activities but not growth. During this phase, the bacteria increase in size, store nutrients, and synthesize materials necessary for replication, before the start of exponential growth (Książek, 2010; Rolfe et al., 2012). In the logarithmic phase, cells are dividing by binary fission and doubling in numbers after each generation time. The log phase is characterised by several rounds of DNA synthesis, transcription, and translation, to synthesize key macromolecules. Bacteria replicate at a constant rate and the logarithm of the bacterial population rises linearly with time. The growth rate is strain-specific and can happen with a doubling time as short as 20 min for E*scherichia. coli* (Książek, 2010; Rolfe et al., 2012). Progressively, there is a decline in available food substrates and nutrients, and waste products start to accumulate, bacterial cell growth reaches a plateau or stationary phase. At this point, the number of dividing cells equals the number of dying cells. During this period, the culture has the highest population density and endospore formation starts after cells enter the stationary phase. As a population overcrowding takes place, nutrients become less available, and toxic products increase, the number of dying cells exceeds cell multiplication. The death phase is characterised by an exponential decline in the number of living cells and a slow population growth rate (Rolfe & Daryaei, 2020; Serra et al., 2014).

2.1.5 Spore Structure

The structure of a bacteria spore and vegetative cell are different. Unlike vegetative cells, the spore has several layers and components that are not present in growing cells. Endospores have a hard and robust structure comprising of many layers such as spore coat, outer membrane, cortex, germ cell wall, inner membrane, core, and sometimes outer exosporium as illustrated in Figure 3. The core is the innermost layer of intact spores consisting of nucleic acids, i.e., DNA, RNA, small acid soluble proteins associated with DNA, enzymes, Ca-DPA and moisture content. Inner membrane surrounding the core region is composed of lipids, act as barrier to many undesirable substances such as toxic chemicals. Inner membrane is further surrounded by germ cell wall composed of peptidoglycan (Setlow, 2008, 2010; Zhang et al., 2010).

The cortex region is located outside the germ cell wall and has a different peptidoglycan composition than vegetative cells. This cortex is surrounded by an outer membrane that plays a unique role in spore formation. The outer membrane is protected from lytic enzymes and other chemical compounds by a protein-based spore coat. In most species, the spore coat is the

outermost layer, whereas in others, the coat is surrounded by an outer layer known as exosporium (Delbrück et al., 2021; Henriques & Moran, 2007; Setlow & Christie, 2021).

The exosporium varies in different species. For instance, in many species, including *B. subtilis*, the exosporium is less prominent and hence the coat is the outermost spore structure while in other species, such as the pathogenic organisms *B. anthracis* and *B. cereus*, the spore is enclosed in an additional layer called the exosporium (Black et al., 2007; Henriques & Moran, 2007). The unique structural properties of spores preserve genetic material and allow them to resist radiations, very low and high range of temperature and pressure, toxic chemical compounds, and several other extreme unfavourable environmental conditions.

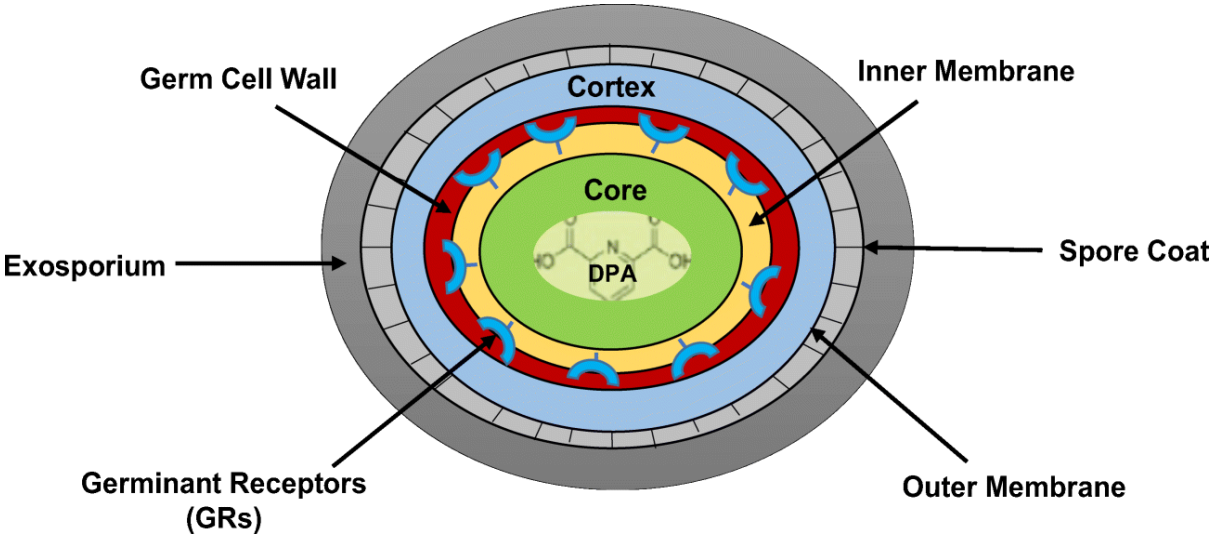


Figure 3: Spore structure. The endospore layers include the exosporium, coat, outer membrane, cortex, germ cell wall, inner membrane and central core (Paredes-Sabja et al., 2011).

2.1.6. Sporulation

Sporulation is an adaptive response that may initiate a response to nutrient deprivation, harsh environmental conditions, or high population densities (Errington, 2003; Kloosterman et al., 2016; Logan et al., 2009). Sporulation is a survival strategy that allow cells to survive in a dormant state. The cells undergo morphological changes during sporulation as the spore develops and matures.

The sporulation process begins with an asymmetric cell division, which produces two distinct cell types: a mother cell and a forespore (the smaller compartment, also known as a prespore). The mother cell eventually dies due to a process known as programmed cell death, whereas the

forespore develops into a spore, resulting in two distinct outcomes for the two cells. Shortly after asymmetric division, cell-specific transcription factors establish two parallel gene expression programs in each compartment. Accurate inter-compartmental communication, as well as regulatory interactions between the forespore and mother cell, are required to regulate the spatial and temporal progression of the developmental process.

After asymmetric division, the remainder of the forespore chromosome, or the origin-distal region, is dragged into the forespore. As a result of the forespore being ingested by the mother cell in a process known as engulfment, the forespore is released as a free protoplast in the mother cell. The cortex, which is made up of modified peptidoglycan, is formed between the two membranes that enclose the forespore. At least 70 different proteins combine to form the complex structure known as the coat (black), which surrounds the forespore surface. After mother cell lysis, the mature spore is released into the environment. B. subtilis cells can remain dormant for a long time, but spores will resume vegetative growth in the presence of tiny molecules (such as single amino acids, carbohydrates, or peptidoglycan fragments) (De Hoon et al., 2010; Fimlaid & Shen, 2015). The process is illustrated in Figure 4.

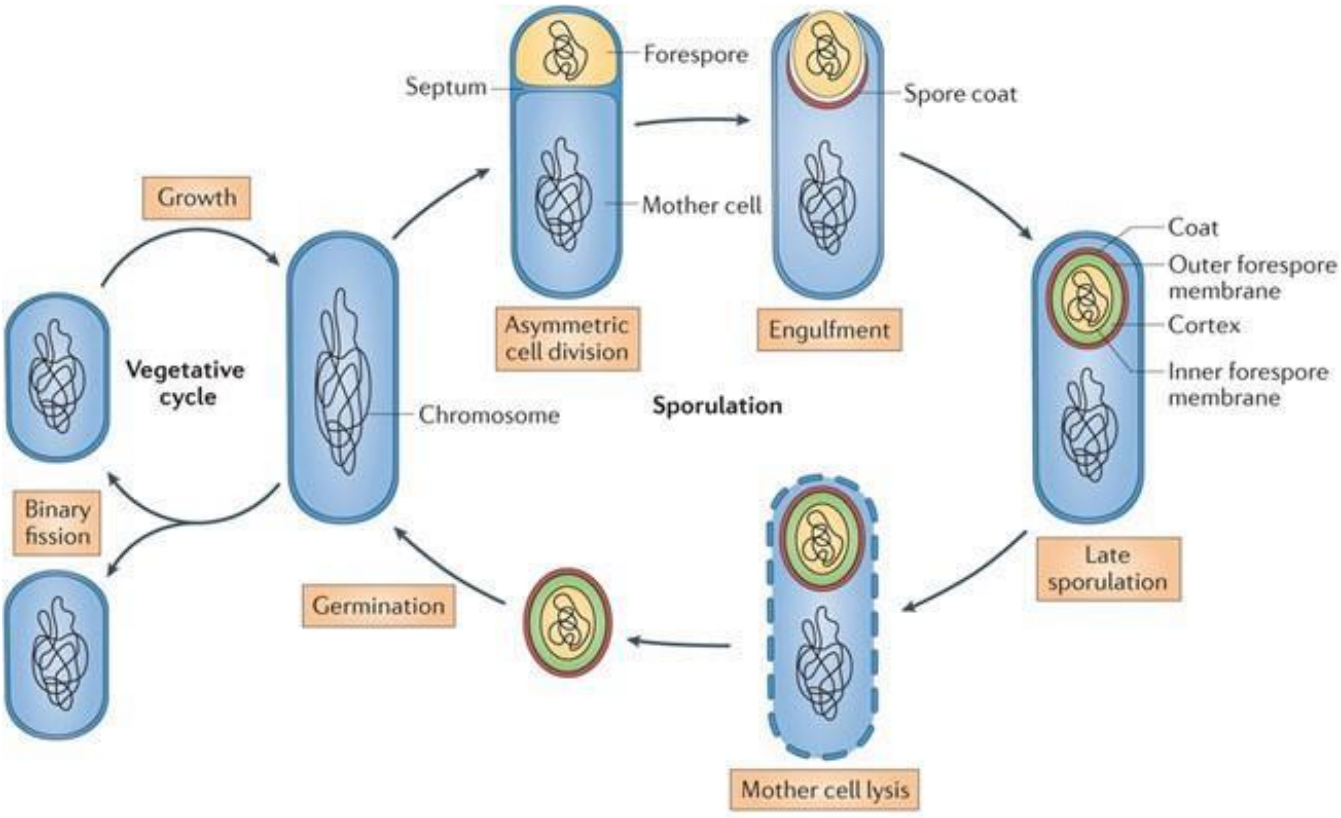


Figure 4: The key stages of sporulation (McKenney et al., 2013)

2.1.7. Germination and outgrowth

The break of spore dormancy due to the availability of favorable conditions may trigger spore activation, germination, and outgrowth (Olguín-Araneda et al., 2015; Paidhungat & Setlow, 2000). Dormant spores retain significant resistance to environmental stresses such as heat, radiation, chemicals, and pH extremes. In the presence of nutrients and other germinants, there is the exit from dormancy and the regain of a vegetative growth cycle to generate growing vegetative cells (Paredes-Sabja et al., 2011; Ross et al., 2003; Setlow, 2010).

Germinants are molecules that cause spores to germinate. They are divided into two types: nutrient and non-nutrient. Germination begins in different ways for different types of germinants. Germination and outgrowth refer to the process by which a dormant spore becomes a metabolically active proliferating cell. This is schematically summarised by (Setlow, 2003) in Figure 5. Germination can be described as a period starting with the addition of a compound (germinant) or a physical treatment that can initiate spore germination, followed by numerous irreversible processes that makes the spore loses its dormant properties, such as core dehydration, refractivity, and heat/chemical/irradiation resistance (Paidhungat et al., 2002). Activation is a related process, although assumed to be reversible, that may act prior to germination, potentiating the spores to germinate at favourable environmental conditions (Løvdal et al., 2011; Setlow, 2014). When germination receptors (GRs) on the inner membrane of the spore recognize germinating molecules (amino acids, sugar, and nucleosides), activation begins in response to nutrient availability (Cho & Chung, 2020). According to Trunet e(2017), spore rehydration occurs during activation, releasing the CaDPA that was previously trapped in the spore core (Trunet et al., 2017). Cortex lytic enzymes (CLEs), such as SleB and CwlJ, the two main CLEs in *Bacillus sp.*, then hydrolyze the cortex. The core can expand and finish rehydrating to about 80% of spore weight thanks to cortex hydrolysis (Setlow, 2014). With core hydration, spores quickly lose their strong resistance to moist heat and chemicals. As the inner membrane that surrounds the centre swells, the spore becomes permeable. Low inner membrane permeability during the quiescent period prevents tiny molecules from entering the core (Sunde et al., 2009). Ultimately, the decrease of spore refractivity results from the hydration of the spore and the release of CaDPA (Figure 5). During outgrowth, metabolic activity and macromolecule synthesis are resumed. The proteinaceous coat and exosporium, the two outermost layers, are where the spores expand up and break free (Abhyankar et al., 2013; Trunet et al., 2017). This process results in the development of a fresh, multiplication-capable vegetative cell. Morphological changes accompany the transition of a germinated spore into a vegetative cell. The cell enlarges, the new cell emerges from the exosporium and coat,

and the restoration of metabolic activity signifies a profound physiological change (Pandey et al., 2013). Very diverse transitions occur from dormant spores to germinated spores, then from germinated spores to vegetative cells (Leggett et al., 2012). Since the germination and outgrowth behaviour depends on the molecular reserves formed during spore formation, this variability may be caused by variations in the expression of genes controlling sporulation and/or environmental circumstances among the population of cells during sporulation. Due to this heterogeneity, spore dehydration rates, the amount of germination-related enzymes present, and the number of germinant receptors established during sporulation are all variable (Chastanet et al., 2010; Lenz & Vogel, 2015). Heat resistance, germination, and growth capacity are thus impacted by this variability (Hornstra et al., 2006; Setlow, 2014).

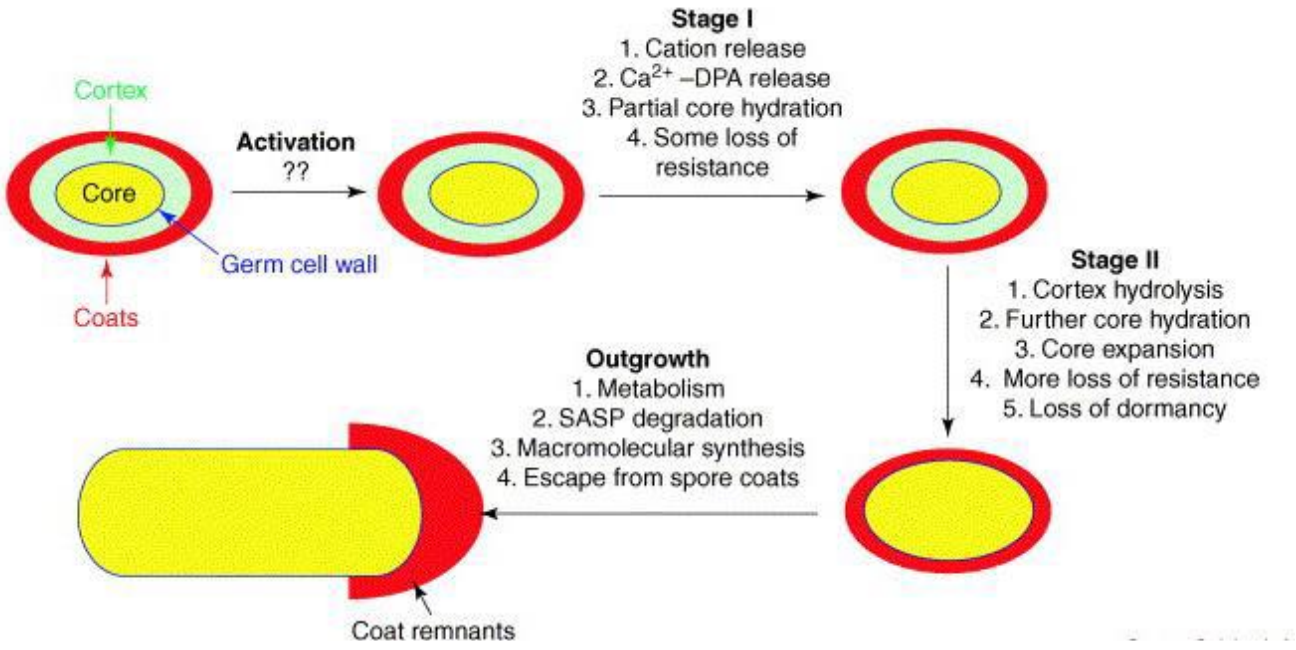


Figure 5: The stages involved in the transition of a dormant spore to a metabolically active proliferating cell; activation, germination (stage I and II) and outgrowth (Setlow, 2003).

2.1.8 Inactivation Kinetics

According to (Shearer et al., 2002), there is an exponential relationship between the number of surviving microorganisms and the time of exposure to a constant temperature. The relative heat

resistance of different microorganisms can be compared and expressed by the D_T parameter or commonly referred to as the D-value. D_T is the amount of exposure time needed to lower the bacterial population by one log10 cycle at a specific temperature (Sörqvist, 2003). D_T, then, is the amount of time required to render 90% of the bacterial population inactive. The link between the D_T value and treatment temperature is comparable. The z value therefore represents the number of degrees needed to raise the treatment temperature in order to decrease the D_T value by one Log10 cycle (Gould, 2006). Thermal treatments, which can inactivate vegetative cells, bacterial spores, yeast, and molds, are the most conventional approach for microbial contamination control.

The type of inactivated microorganism is largely determined by the strength of the treatments used. The degree of heat resistance of different microorganisms is similarly influenced by their structure, composition, and resistance mechanisms. For example, heat resistance varies significantly between vegetative cells and spores of the same bacterial species (Lv et al., 2021). Bacterial spores are thus more heat resistant, and they can even withstand pasteurization treatments (D_T values frequently exceeding 1 minute at 100 °C). Vegetative bacterial cells, on the other hand, are relatively heat sensitive (Løvdal et al., 2011).

In a study by (Byrne et al., 2006), a D-value of 50 °C at 33 min was enough to kill vegetative cells, while 85 °C was required to inactivate the spores of the microorganism. In recent years many refrigerated processed foods of extended durability (REPFED) are produced at temperatures between 65 and 95°C. These temperatures may eliminate the competing vegetative flora but are inadequate to kill or inactivate bacterial spores. These are sublethal temperatures which may potentially create favorable conditions for spore inactivation (Oomes et al., 2009). Factors that affect the inactivation kinetics include bacterial strains, age of the culture, food composition (fat, NaCl, pH and aw), and physiological state of the organisms (Liu et al., 2011; Lv et al., 2021). The effect of food composition on inactivation kinetics was evaluated in an experiment by (E. Evelyn & F. V. Silva, 2015), and D_{90} °C-values of 3.15 min and 1.03 min were reported for *B. cereus* ATCC 11778 spores in skim milk and beef slurry, respectively. D and z values also differ between bacteria strains and show viable D and z values in different media. Desai and Varadaraj (2010) discovered a z-value of 25 °C for *B. cereus* CFR 1521 spores in saline, while another strain of *B. cereus* (F 4810) showed a z-value of 16.7 °C under the same cultured conditions (Desai & Varadaraj, 2010). Even though thermal treatments can effectively inactivate *Bacillus* spores, extreme temperatures can cause component degradation, resulting in a loss of structure, taste, and texture aroma (Holdsworth and others 2008).

Table 2: Reported D-values for* spores *of different bacterial strains in different matrices (Soni et al., 2016).

Bacterial strain	**D-value (min)**	**Reference**
B. cereus in pork luncheon meal	D_{100}= 2.02	Byrne and others (2006b)
B. cereus in infant formula with 10% total solids	D_{100}= 1.8	Stoeckel and others (2013)
B. cereus in beef slurry	D_{100} = 0.42	Evelyn and Silva (2015)
B. licheniformis in UHT milk	D_{100} = 2.37	Janˇstov´and Luk´aˇsov´a (2001)
B. subtilis in phosphate buffer	D_{100}= 0.5	Jagannath and others (2005)
B. cereus in water	D_{90} = 2.5	Stoeckel and others (2014)

Mathematically, the decimal reduction time (D-value) can be expressed by the following equation:

$$D = \frac{\mathrm{t}}{\mathrm{logN0} - \log \mathrm{N}}$$

According to this equation, 't' represents the heating time; 'N_0'is the initial number of microorganisms and 'N' is the final number of microorganisms after heating. The D-value can also be represented by plotting the log concentration of microorganisms (cfu/ml) against the appropriate heat treatment time to obtain a linear curve (Figure 6 and Figure 7). This curve is known as a survivor curve. By using the slope of the line, D- value is calculated by using the Equation:

$$D = \frac{-1}{a}$$

Here ‘a’ represents the slope of the line (Soni et al., 2016).

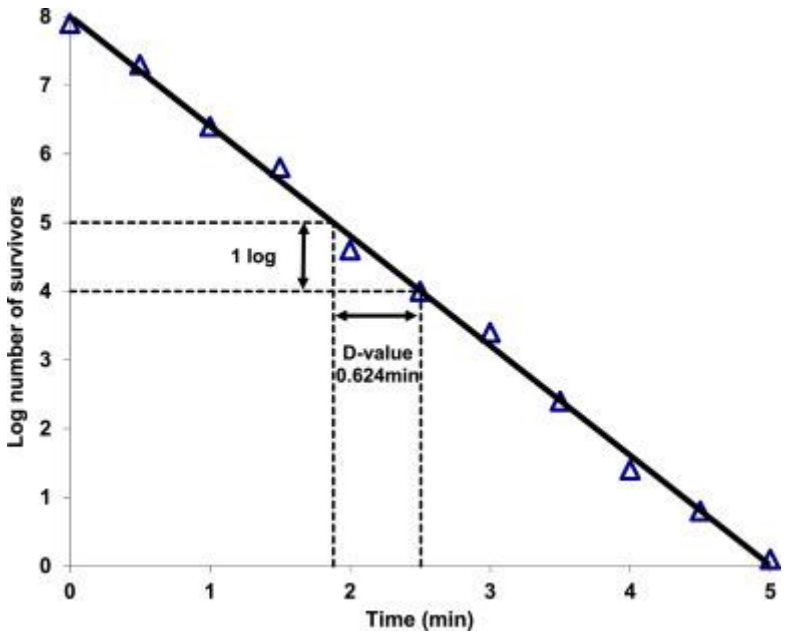


Figure 6: A graphical representation of D value. For an isothermal inactivation, the D value is the time required to obtain a 1 log reduction in viable bacteria (Stringer & Metris, 2018).

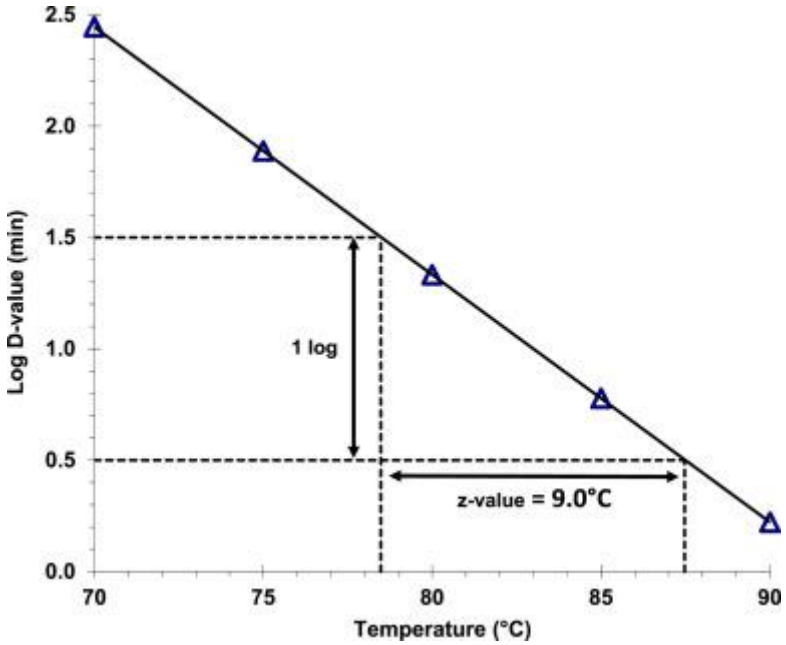


Figure 7: A graphical representation of z value. If the logarithm of D is approximately a linear function of the heating temperature, the z value is the increase of temperature required for a 1 log reduction in D value.

Heat resistance among different bacterial populations vary in food products. Some bacteria have higher heat resistance and bacteria in this category usually form spores. Heat treatments such as pasteurisation are applied to kill vegetative bacteria while spore-forming bacteria require sterilisation to inactivate spores(Peck et al., 2008). With reference to European guidelines, the minimum heat treatment for pasteurizing a sous vide food should be the equivalent of heating at 70 °C for 2 minutes at the slowest heating point. This is based on ensuring a 6 log (6D) reduction of *L monocytogenes*. On the other hand, psychrotrophic spore forming pathogens including Group II *C. botulinum* and psychrotrophic strains of *Bacillus cereus* require heat treatment of 90 °C for 10 min or a process of equivalent lethality. This is based on a requirement for a 6-log inactivation of spores of Group II *C. botulinum* to avoid potential growth and toxin production by this organism. Mesophilic spore forming bacteria including Group I *C. Botulinum* (proteolytic *C. botulinum*), mesophilic *B. cereus* and *C. Perfringens* require thermal inactivation based on the 12D concept where foods are subjected to a heat treatment that will reduce the number of viable Group I *C. botulinum* spores by factor of 10^{12}. The heat resistance parameters generally applied to Group I *C. botulinum* are a $D_{121.1}$ °C value of 0.21 min and a z value of 10 °C. The time to achieve a 12-log reduction at 121.1 °C would be 2.52 min (12 x 0.21). This time is gives the "botulinum cook" for sterilisation (Stringer & Metris, 2018). In a study by (Byrne et al., 2006), a 6-log reduction

of *B. cereus* and *C. perfringens* vegetative cells was achieved when pork luncheon was mildly cooked at 70 °C for 12 s and 1.3 min respectively. To achieve a similar log reduction of *B. cereus* and *C. perfringens* spores, the pork luncheon was heated for 36 s at 105 and 110 °C, respectively. The *Table 3* provides further information about the D-values and temperatures at which *B. cereus* and *C. perfringens* vegetative cells and spores are inactivated.

Table 3: The D-values (min) for B. cereus and C. perfringens vegetative cells and spores at different temperatures (Byrne et al., 2006).

Temperature (°C)	**Cocktail**	R^2	***D*-value (min)**
50	*B. cereus* vegetative	0.98	33.2
55	*B. cereus* vegetative	0.98	6.4
60	*B. cereus* vegetative	0.96	1.0
85	*B. cereus* spores	0.99	29.5
90	*B. cereus* spores	0.98	10.1
95	*B. cereus* spores	0.98	2.0

2.2 Food Processing

The concept of food processing dates back into prehistoric times, where foods were usually subjected to heat to modify and extend the shelf-life foods. In past decade, the focus of food processing has changed from traditional methods of cooking to more industrialized methods, with initially an emphasis on preservation and later on (1920–1930s) safety (microbial safety) and quality issues, especially nutritional quality issues after the Second World War (Van Boekel et al., 2010). Traditionally, thermal processes have been used extensively in food technology. Thermal treatment of foodstuffs induces several biological, physical and chemical modifications, leading to sensory, nutritional and textural changes (Kubo et al., 2020). Also, some undesirable reactions are known to occur, leading to loss of nutritional value and the formation of potentially mutagenic and carcinogenic molecules which generally reduces consumer preferences for foods processed under these conditions (van Boekel et al., 2020). In view of this, many processing technologies have emerged to alter and replace the prevailing processing technologies so that foods with better quality to suit consumer preferences can be manufactured. In last few decades, the need for foods with milder processing techniques have

increased, which has led to the development of different novel processing technologies to improve the physico-chemical properties of foods by minimizing processing and thermal degradation impacts. Examples of these novel technologies are pulsed electric fields (PEF), ohmic treatments, high-pressure processing (HPP), ozone treatment, non-thermal plasma/cold plasma (NTP), ultrasound technology, hurdle etc (Khouryieh, 2021). Since the quality of food largely depends on the processing techniques applied, there is the need to understand these processing techniques and their impact on the quality and safety of foods. Figure 8 provides an overview of novel thermal and non-thermal food processing technologies.

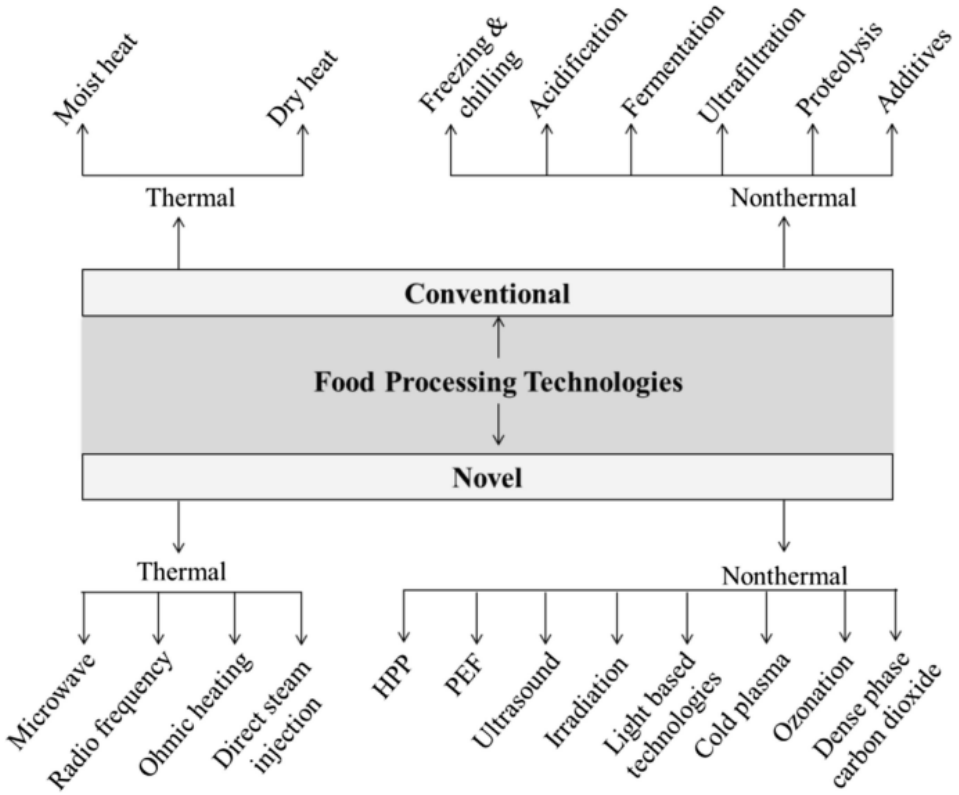


Figure 8: Overview of novel thermal and non-thermal food processing technologies. Food Engineering (Barbosa-Cánovas et al., 2020).

2.2.1 Thermal Processing

Thermal processing still remains the most prevalent technology in food processing even in the present time (Misra et al., 2017). Till date, thermal technology is commonly used in the processed food market in every aspect of operation, equipment, automation, food safety and quality (Pankaj, 2015). Historically, thermal processing dates back to many centuries and has evolved in many forms which are used in modern industrial processes (Featherstone, 2012). Pasteurization and Sterilization are some thermal treatments applied to food to extend their

shelf-life. Pasteurisation is a very common thermal procedure in food industry, applied to many food products (milk, cream, juice, beer, etc.) to prolong their shelf-lives. Pasteurisation is carried out by heating the product to the pasteurisation temperature, and then holding that temperature (usually less than 75°C) during a fixed time (Silva, 2019). Pasteurisation temperatures are enough to eliminate non-spore forming bacteria, yeasts and molds but spores of spore-forming bacteria may survive. The recognition of Clostridium botulinum spores as target microorganisms in low-acid foods (pH ≥ 4.6) paved way for the use of sterilisation in food processing to kill spores of spore-forming bacteria (Setlow, 2019). Sterilisation is based on the concept of 12-D or botulinum-cook, as the minimum thermal processing requirement to achieve commercial sterilization, by causing 12 decimal reductions of C. botulinum spores, the most resistant pathogenic bacterial spore (Featherstone, 2012). The objective of this thermal processing technique is to attain a target sterilization temperature (121°C for 3 minutes) to reduce the number of spores by 12 log10 (Sevenich et al., 2013). As stated earlier, thermal treatments affect the taste and nutritional quality of foods even though its application prolongs the shelf-life of foods (Aghajanzadeh et al., 2022; Liu et al., 2022). The impact of thermal treatments on food has led to the emergence of many novel and non-thermal technologies with prime focus of enhancing the safety and quality of foods with better consumer preferences (Chacha et al., 2021).

2.2.2 High Pressure Processing (HPP)

In recent years, high-pressure technology is acknowledged as one of the novel food processing technologies with the promising potential of producing better value added products with improved nutritional and sensory qualities. HPP treated foods remain the choice of many consumers due to their fresh-like attributes with safety and prolonged shelf-life ensured (Abera, 2019; Farkas & Hoover, 2000; Wang et al., 2015). According to (Rastogi & Knorr, 2013) the advantages of this technology include the following.

- HPP enables food processing at ambient temperature or even lower temperatures
- It enables instant transmittance of pressure throughout the system, irrespective of size and geometry, thereby making size reduction optional, which can be a great advantage
- It causes microbial death whilst virtually eliminating heat damage and the use of chemical preservatives/additives, thereby leading to improvements in the overall quality of foods

- It can be used to create ingredients with novel functional properties.

High pressure processing is a non-thermal food processing method in which food is hermetically sealed in a flexible container and subjected to a high pressure of 100-600 MPa at room temperature. To achieve pasteurization, a liquid (typically water) is used as the pressure transfer medium, while the interior and surface of the food are subjected to even pressure (Balasubramaniam et al., 2015; Huang et al., 2017). According to research, pressures ranging from 300 to 600 MPa can inactivate yeasts, molds, and the majority of vegetative bacteria, including the majority of infectious food-borne pathogens (Agregán et al., 2021; Silva, 2019). More information about spores of different *B. cereus* strains and their log reductions by HPP application can be found in *Table 4*.

Table 4: Inactivation of spores of different B. cereus strains by HPP (Evelyn & Silva2019).

Strains	Pressure (MPa) and holding time	Processing temp. (°C)	Log reductions	Reference
B. cereus ATCC 9818 in cooked rice	600, 4 min	84	7.0	Daryaei et al., (2013)
B. cereus NZ 6, NZ5, NZ 4 (NCTC 8035), NZ 3, NZ 7 in Skim milk	600, 1 min	72	3.8, 3.9, 4.2, 4.4, 4.5	Robertson et al., (2008).
B. cereus ICMP 12442 (ATCC 9139) in beef slurry	600, 15 mins	70	4.6	Evelyn and Silva (2016a)

B. cereus NCFB 578, 1031 in milk	600, 15 mins	Room Temperature (RT)	< 0.5	Mc Clements et al., (2001)
B. cereus ATCC 9139 in cheese	400, 15 mins	RT	<0.5	Lopez (2003)

The application of pressure instead of heat leaves small molecules such as many flavour compounds and vitamins intact. In microbial spore inactivation, bacterial spores can only be killed by very high pressures >1000 MPa (Lv et al., 2021). However, at pressures of 100-400 MPa, bacterial spores can be stimulated to germinate. At these pressures, nutrient-like physiological germination is triggered where the nutrient receptors in the spore inner membrane are activated by pressure, prompting the germination process. Germinated spores can then be killed by relatively mild heat treatments or mild pressure treatments but in most cases some spores can survive this treatment. High pressures (>400 MPa) can be used to induce the outgrowth of endospores by direct opening of the dipicolinic acid channels, resulting in its release to the external media (Reineke, Ellinger, et al., 2013). HPP has a disruptive action on the vegetative cells of bacteria. In fact, HPP is known to be responsible for changes in the microorganisms' membranes, resulting in leakage of the inner cell content and interference on nutrient uptake mechanisms (Sevenich et al., 2014). Many literature studies have explained high-pressure effects on cytoplasmic proteins and nucleic acid in gram positive pathogens such as *B. cereus*. Reports show that HPP inhibits protein synthesis by dissociating ribosomes and affecting DNA synthesis and proteins expression, which ultimately activates stress response in bacteria. An alternate mechanism is that HPP induces oxidative stress and creates some reactive oxygen species to very low levels but high enough to kill bacteria. Finally, HPP denatures intracellular proteins and enzymes which affects bacterial metabolism (Gänzle & Liu, 2015). Figure 9 illustrates schematically the process of microbial inactivation under pressure.

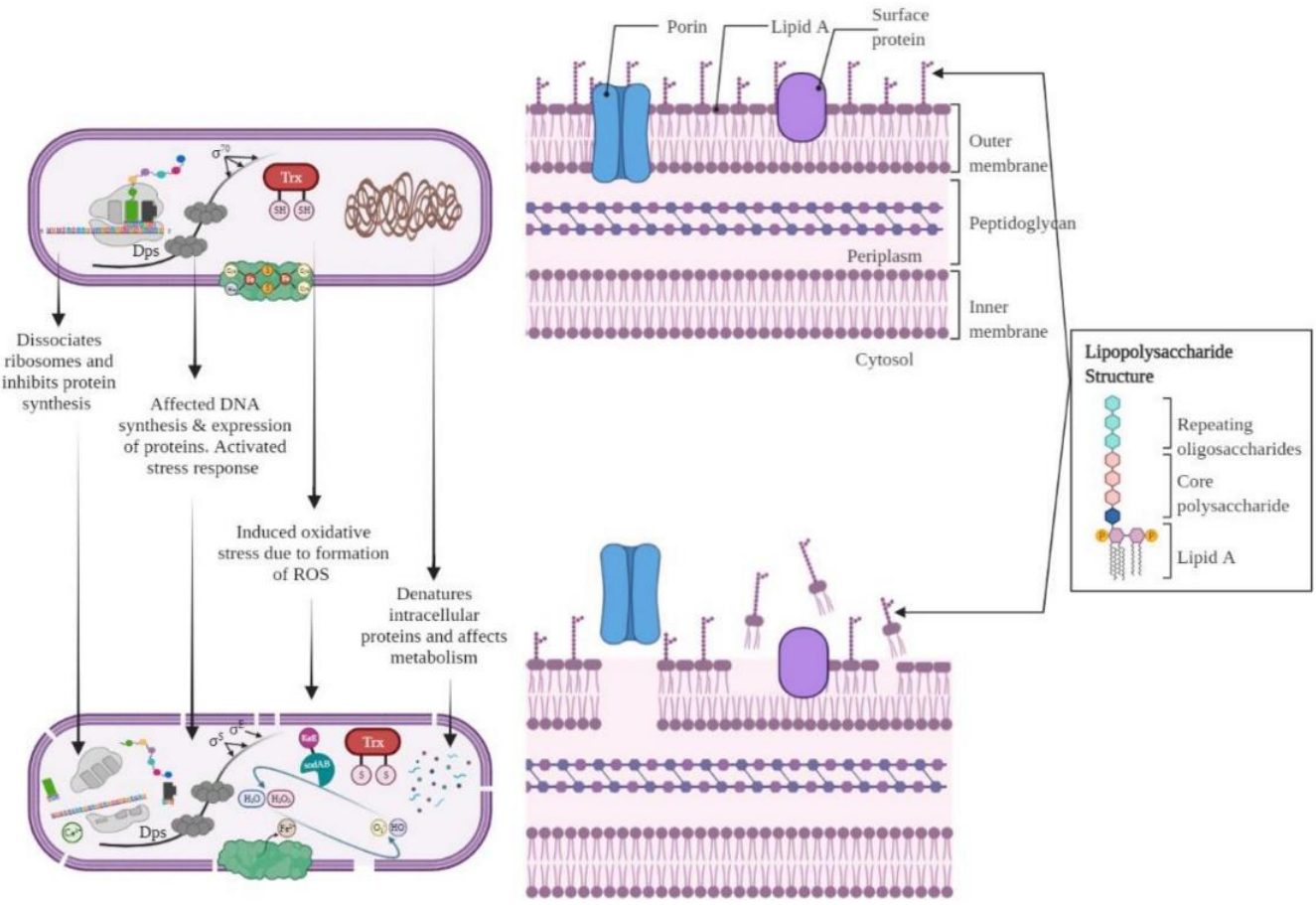


Figure 9: Schematic representation of the process of microbial inactivation under pressure (Gänzle & Liu, 2015).

To inactivate a Gram-positive bacteria, a more intense pressure treatment is required than inactivating a Gram-negative bacteria. This is due to the rigidity of teichoic acids present on the peptidoglycan layers of Gram-positive bacteria (Di Pinto et al., 2013; Heinz & Buckow, 2010).

Different microorganisms react with different degrees of resistance to HPP treatment, and indeed there can be vast HPP sensitivity among bacterial species and even strains (Sehrawat et al., 2021). While some bacterial spores are extremely resistant to HPP treatments, a combination of heat and pressure treatments have been studied, and although these methods achieve spore inactivation to some extent, total efficacy is determined by a variety of factors such as bacterial species, number of treatment cycles, pH, pressure, processing time, and temperature (Considine et al., 2008; Torres & Velazquez, 2005). HPP at 400-600 MPa for 10-15 minutes at room temperature resulted in a 1.0 log reduction of *B. cereus* spores according to a study by (López-Pedemonte et al., 2003). On the other hand, HPP alone is not

enough to inactivate *B. cereus* spores. *Bacillus subtilis* spores, for instance, have been found to withstand processing conditions of up to 1200 MPa at room. As a result, heat-assisted HPP (HPTP - high pressure thermal processing) at temperatures greater than 50 °C is required in addition to cold-chain management for the inactivation of resistant spore-formers. According to research, the temperature and pressure of HPTP work together to enhance microbial spore inactivation (E. Evelyn & F. V. Silva, 2015; Silva, 2016). *Bacillus* spores must generally be inactivated at pressures of at least 500-600 MPa and temperatures of at least 60 °C-85 °C. (Van Opstal et al., 2004) reported > 5 log spore inactivation for four strains of *B. cereus* in milk after 500 MPa, 60 °C, and 15 minutes.

2.2.3 Pressure-assisted thermal sterilization (PATS)

The ability of high-pressure processing to inactivate vegetative cells of microbes present in food and still maintain the organoleptic properties of the food makes HPP the ideal choice of many food processing industries (Aldrete-Tapia & Torres, 2021; Daryaei et al., 2013; Erkmen & Doğan, 2004; Ross et al., 2003). However, HPP alone is insufficient to kill bacterial spores (Balasubramaniam et al., 2015; Martínez et al., 2007). Many research studies have revealed the coupled potential of heat and pressure to inactivate bacterial spores (Sevenich et al., 2013). This process is described as pressure-assisted thermal processing (PATP) or high-pressure thermal sterilization (HPTS). This process involves applying elevated pressures (up to 700 MPa) and sub-retorting temperatures (90-120 °C) to preheated food at the same time. This technology has the advantage of minimizing the severe impact of thermal treatments encountered during conventional processing techniques. This is due to the ability of this process to instantly raise the temperature of the test sample due to the heat of compression caused by the rapid pressurisation of the sample (Ahn et al., 2015; Martínez-Monteagudo & Balasubramaniam, 2016). There is a decrease in temperature after decompression throughout processing. Shorter processing times with little influence on the nutritional qualities of the food is attributed to this process (Balasubramaniam et al., 2015; Barbosa-Cánovas et al., 2014; Sevenich et al., 2014).

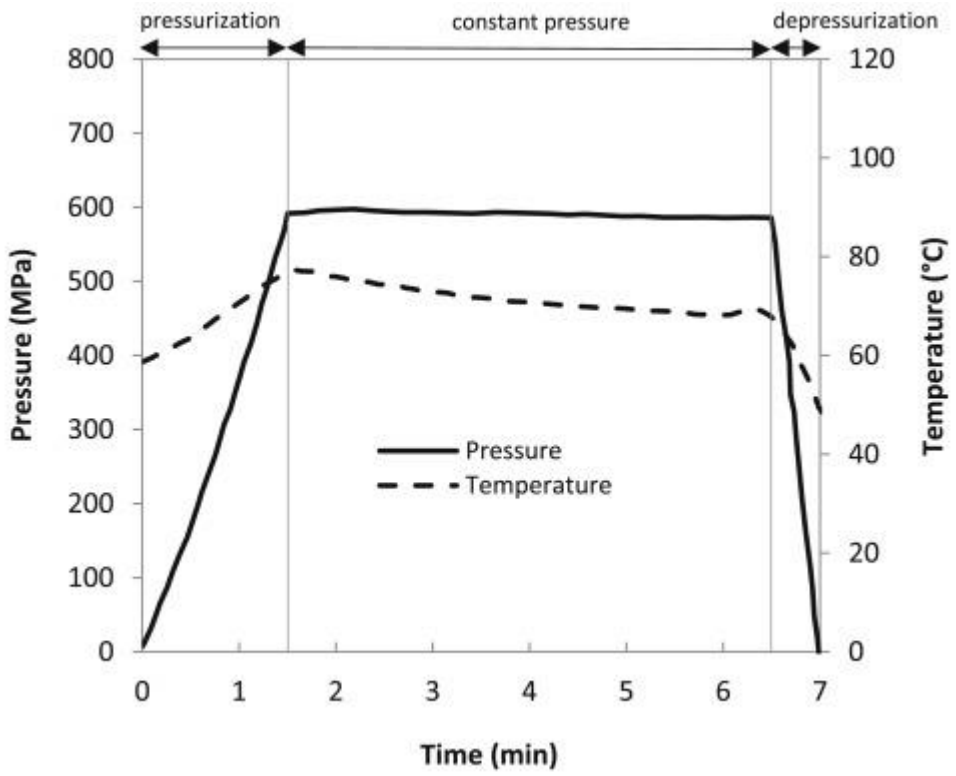


Figure 10: Temperature and pressure profiles during PATS. HPTP process at 600 MPa and 70 °C for 5 minutes demonstrating the three phases of the process cycle (pressurization, constant pressure, and depressurization) (Silva, 2019).

It has been found that the PATS process consists of six major stages: (i) vacuum packaging and product loading, (ii) preheating to target temperature, (iii) product equilibration to initial temperature, (iv) product temperature increase to pressurization temperature via compression heating, (v) product temperature decrease during decompression, and (vi) product cooling to ambient temperature (Barbosa-Cánovas & Juliano, 2008). Prior to this process, air is removed from the food, and the product is vacuum packed in a flexible plastic film. The prepacked product is also preheated to the required temperature prior to PATS. The pressure chamber is warmed to minimize heat loss to the environment for effective heat treatment. The packed food is then loaded into the pressure chamber, which is then closed and sealed. The pressure cycle starts, and a pressure transferring fluid set at a specified temperature is pumped into the chamber, allowing the remaining air to escape through a vent valve. The vent valve is closed, and more fluid is forced into the chamber via hydraulic pumps. The product is then cooled after the process by keeping on ice to avoid thermal degradation. (Barbosa-Cánovas et al., 2014; Barbosa-Cánovas et al., 2020).

During a PATS process, the amount of heat received is determined by three main conditions target preheating/equilibration temperature, selected pressure, and pressure/temperature holding time as described in the Figure 10 above. Furthermore, there are other inherent factors such as the presence of lower temperature sites in the vessel, pressure come up rate, pressure holding, decompression rate, and food or package properties. These factors should be taken into account when evaluating sterilization performance (Barbosa-Cánovas et al., 2014; Daryaei et al., 2013).

2.2.4 Inactivation of bacteria spores by pressure-thermal processing

The resistance of spores to pressure may be contributed by sporulation temperature and the composition of the sporulation media. Reports by (Delbrück et al., 2021) showed that sporulation temperatures affected the resistance of *B. subtilis* spores to pressure and also to resulted in a significant alteration in the spore coat and cortex composition (Delbrück et al., 2021; Reineke & Mathys, 2020; Setlow & Christie, 2023). When compared to spores sporulated at lower temperatures, higher sporulation temperatures increased the responsiveness of nutrient germinant receptor (nGR) to moderate pressure (150 MPa). Despite lower levels of nGRs, sporulation temperature affects fluidity and other aspects of the inner spore membrane composition (Black et al., 2008). In contrast to thermal inactivation, the inactivation of spores under HP combined with thermal treatments have been explored. Although, there have been contrasting views about the exact mechanism of spore inactivation under pressure-thermal processing, a two-step inactivation mechanism has been proposed. These steps include the release of DPA, which is accompanied by a loss of heat resistance causing spore inactivation (Reineke, Ellinger, et al., 2013). According to this mechanism, nutrient receptors are activated at ambient pressures to release pydridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) from the spores.

Spore germination is triggered by the partial rehydration of the spore core and this results in a reduction in heat resistance of the partially germinated spore (Setlow, 2003, 2010). At this point, the pressure-induced germination of spores allows a subsequent inactivation of germinated spores by mild-heat or pressure (Sunde et al., 2009). Margosch et al. (2004) reported that endospore resistance to a combined HP (>600 MPa) and temperature (>60°C) treatment is dependent on the spore's ability to retain DPA as well as the heat resistance of the DPA-free spores by testing 19 *Bacillus* strains. Various inactivation methods have also been proposed based on research by (Knorr et al., 2011; Reineke, Mathys, et al., 2013). These

researchers proposed a three-step process that includes germination, an unknown step, and an inactivation step (Reineke, Ellinger, et al., 2013).

Table 5: Log reductions of B. cereus spores by different pressure-thermal processing (Soni et al., 2021).

Species of *B. cereus*	**Substrate**	**Treatment condition**	**Log reduction**	**Reference**
ATCC 9139, ATCC 21	Milk	600 MPa, 70℃, 20 min	4.0	Evelyn & F. V. M. Silva, 2017
ATCC 1778		600 MPa, 70℃-78℃, 2 min	6.0	Evelyn & F. V. M. Silva, 2018
ATCC 1778	milk	600MPa ,70 °C, 15 min 200MPa ,70 °C, 40 min	3 3.6	(Evelyn & F. V. M. Silva, 2015)
ATCC 9139	Cheese	400 MPa, 30 °C, 15 min	<0.5	(López-Pedemonte, Roig-Sagués, Trujillo, Capellas, & Guamis, 2003)
ATCC 9818	Cooked Rice	600 MPa, 85 °C, 4 min	7	(H. Daryaei, Balam, & Legan, 2013)

2.3 Preservatives

Many factors can influence the quality and safety of foods especially during storage. Food preservatives are used to prevent the spoilage of foods and extend their shelf-life (Zhang et al., 2021). They are compounds that have been added on purpose to avoid deteriorative reactions and thus protect the quality and shelf life of foods and beverages. Food preservatives can be derived from natural sources or synthesized. They can be extrinsic (intentionally added), intrinsic (normal constituent of food), or developed (produced during fermentation) (Da Cruz

et al., 2021). Preservatives are used in limited quantities and at low levels, ranging e.g. from parts per million (ppm) to 1-3% by weight (Dias et al., 2022). The functions of many food preservatives are multifaceted including extending shelf life by inhibiting oxidation, browning, or enzymatic reactions and ensuring microbial safety by inhibiting or killing pathogens. Several factors must be considered when selecting the appropriate food preservative including the type of microorganism present (pathogen or deteriorative), pH, composition, physical state, product shelf life required, use, and application. The effectiveness of a food preservative also depends on several factors such as the concentration of inhibitor, kind, number, and age of microorganisms (older cells more resistant), temperature, time of exposure (if long enough. some microbes can adapt and overcome inhibition), and chemical and physical characteristics of food (water activity, pH, solutes, etc.) (Davidson et al., 2012; Taylor & Doores, 2020).

Examples of inorganic preservatives include sodium chloride (NaCl), nitrate and nitrite salts. NaCl lowers water activity and causes plasmolysis by withdrawing water from cells (Marshall et al., 2016). In a study by (Kim et al., 2017), the effect of different concentrations NaCl on *B. cereus* growth in salted shrimps was studied. In shrimp treated with 2, 5, 10, and 15% NaCl, the overall *B. cereus* count decreased significantly to 6.59 (0.18 log reduction), 4.82 (0.66 log reduction), 3.93 (0.43 log reduction), and 2.25 (0.18 log reduction) log CFU/g, demonstrating the impact of increasing NaCl concentrations on *B. cereus* growth. The use of nitrites and nitrates as meat curing agents (hams, bacon, sausages, etc.) to inhibit C. botulinum under vacuum packaging conditions has also been studied (Flores & Toldrá, 2021; Lee et al., 2018).

Herbal extracts, spices and essential oils have also been explored for their antimicrobial properties (Chaleshtori et al., 2021). Carvacrol, an antimicrobial compound found in the essential oils of oregano and thyme, has been shown to inhibit growth and diarrheal enterotoxin production in *B. cereus* inoculated in brain heart infusion medium (BHI), cooked rice, and mushroom soup (Macwan et al., 2016; Silva et al., 2013; Ultee et al., 2000). Carvacrol was less effective in a food matrix, as evidenced by higher concentrations required to achieve the same effect in food as in laboratory medium, most likely due to interaction with food components (Boskovic et al., 2015; Ultee et al., 2000). Nonetheless, these studies highlighted the potential use of carvacrol for food preservation, increasing the safety of the products. Likewise, bactericidal effects of cinnamaldehyde and thymol against *B. cereus* have been documented (Valero & Frances, 2006; Valero & Salmeron, 2003), as has the development of synergistic effects between carvacrol or thymol and nisin (Churklam et al., 2020; Periago et al., 2001). Furthermore, (Valero & Frances, 2006) discovered that adding cinnamaldehyde or 20 mg thymol to 100 ml of broth with refrigeration temperatures (8 °C) could prevent the outgrowth

of activated spores of the psychrotrophic strain INRA TZ415 for at least 60 days. One of the most commonly used bacteriocins, nisin, is known to have antimicrobial properties against certain bacterial species. It's used as a food additive (E-234) as well as a component of coatings and films. It is used in dairy products (ranging from 100 to 4000 IU/mL), beverages, eggs, and meat (ranging from 400 to 1000 IU/mL), among other things. Recent research indicates that it can be enhanced by synergisms with other antimicrobials (Gharsallaoui et al., 2016; Pajohi et al., 2011). *Bacillus cereus* ATCC 1479-8 spores were tested for decimal reduction times (D-values) in rice and milk (13% wt/vol) supplemented with nisin (25 mg/ml) at cooking and autoclaving temperatures (80 to 120°C). The addition of nisin to milk resulted in a 40% reduction in D-values over a temperature range of 80 to 100°C. At 110°C, the D-value for milk (control) and milk with nisin was 0.86 min. Furthermore, the z-values ranged from 7.32°C (phosphate buffer) to 10.37°C (water) (Munoz, 2018).

2.4 Hurdle Technology

The main aim of hurdle technology is to improve the total quality of foods by application of a combination of preservative factors known as hurdles (Leistner & Rahman, 2020). In this way, the microbial safety and stability of the food is maintained (Singh & Shalini, 2016). The principle behind the hurdle effect was derived from the fact that microorganisms present at the start in a food should not be able to overcome (leap over) the hurdles present during the storage of a product, else the food will spoil or even cause food poisoning. Another key feature of hurdle technology is the disturbance of homeostasis of micro-organisms. Thus, subjecting microorganisms to stress reactions may result in metabolic exhaustion of the microorganism leading to food preservation (Neeha & Kakade, 2014; Singh & Shalini, 2016). Hurdle technology is vital for the preservation of intermediate-moisture foods as well as high-moisture foods. Some important hurdles used in food preservation include temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrite, sorbate, sulfite), and competitive microorganisms (e.g., lactic acid bacteria) (Tapia et al., 2020). However, more than 60 potential food preservation hurdles have already been described, and the list of possible food preservation hurdles is far from complete (Dabas & Khan, 2019; Rahman, 2014). While some hurdles have a positive outcome, others have a negative effect on the foods depending on its intensity. For instance, the pH of fermented sausage which should be low enough to inhibit pathogenic bacteria, but not so low as to impair taste. According to (Leistner, 2000), the use of any single hurdle with high strength or high concentration can have a negative impact on food quality, such as loss of nutrients, texture, and color. Therefore, how these hurdles are intelligently combined is

of great importance to maintain the quality of the food (Tsironi et al., 2020). It is worthy to know that no single factor is responsible for achieving a stable product, but rather the stability may arise from the synergism or combination of several preservative factors known as hurdles (Singh & Shalini, 2016). In an experiment by (Silva, 2016), the effect of combining ultrasonication, mild-heat treatment, and HPP to eliminate *Alicyclobacillus acidoterrestris* NZRM 4447 spores were investigated. Results showed that HPP (200 or 600 MPa, T < 39 °C for 15 min) along with thermosonication (20.2 W/mL, 78 °C for 60 min) reduced *Alicyclobacillus acidoterrestris NZRM 4447* spores by 2.3–4.4 log CFU/g in commercial orange juice. In another experiment, Evelyn et al. (2016) studied and compared the efficacy of 600 MPa HPP in combination with 70 °C for the inactivation of *B. cereus* ICMP 12442 spores in beef slurry to 70 °C thermal processing alone. The HPP-70 °C process improved *B. cereus* spore thermal inactivation in beef slurry, resulting in 4.9 log reductions after 20 minutes vs. 0.5 log reductions for thermal processing (Silva, 2016). Studies by (Valero & Frances, 2006) also reported at least 60 days inhibition of *B. cereus* when the synergistic effect of refrigeration and mild acidification (pH 5.0) with lemon juice on the growth of *B. cereus* in carrot broth was investigated. Le Marc et al., (2021) investigated the effect of pH, alone or in combination with temperature, on the maximum growth rate (μmax) of *B. cereus sensu lato* (Le Marc et al., 2021). A study was undertaken to determine the combined effects of pH, nisin, and temperature on growth and survival of 20 strains of *B. cereus*. The effectiveness of nisin in controlling the growth of psychrotrophic strains of *B. cereus* capable of causing human illness was more pronounced at 8°C than at 15°C and as the pH was decreased from 6.57 to 5.53. Table 6 highlights the use of HPP as Hurdle technology against *Bacillus* spores based on several literature studies (Soni et al., 2016).

Table 6: HPP as Hurdle Technology against Bacillus spores (Soni et al., 2016).

Target	Hurdle Technology	Outcome	Reference
Milk	800 MPa at 40 °C for 5 min and nisin (104 IU/mL)	3.7 log reduction	Black and others (2008
Beef	Pomegranate peel and tamarind extract with 600 MPa at 105 °C for5min	6 log reductions	Devatkal and others (2015)
Cheese	Nisin and 60 MPa at 30 °C for 210 min (germination step) + 400 MPa for 30 min at 30 °C	2.4 log reduction	Lopez- Pedemonte and others (2003)
Cheese from goat milk	Germination treatments of 60 MPa at 40 °C for 210 min + 500 MPa at 40 °C for 15 min	4·9 log reduction	Capellas and others (2000)
Milk and beef	392 MPa for 10 to 15 min at 45 °C in the presence of the emulsifier sucrose laurate (<1%)	3.0 to 5.5 log reduction	Reduction Shearer and Others (2000)

3. MATERIAL AND METHODS

3.1. Preparation frozen stock culture of *Bacillus cereus* NVH 1230-88

The *Bacillus cereus* NVH 1230-88 strain used in this study was obtained from the Nofima culture collection centre (Norway). This strain was chosen because it is psychrotrophic and can grow at refrigerated temperatures. Due to this, we wanted to know at which refrigerated temperature it could grow after pasteurisation to prevent potential food safety hazards. In this study, *Bacillus cereus* NVH 1230-88 will be used in its abbreviated form as *B. cereus* NVH.

Three batches of bacterial stock cultures were prepared and stored using Microbank cryovials (Prolab Diagnostic, Wirral, U.K.). Microbank™ is a sterile cryovial system incorporating porous beads and a special cryopreservative solution made from glycerol.

Each batch of bacterial stock culture was prepared by inoculating the cryogenic fluid of the Microbank vials with a single colony from an overnight growth on LB agar incubated at 37°C, using a sterile loop under aseptic conditions. The stock culture was prepared to a density approximately equivalent to a McFarland standard of 3 or 4. The inoculated fluid was mixed four or five times to emulsify the suspension and to bind the microorganisms to the beads. The extraneous cryogenic fluid was then aspirated using a sterile Pasteur pipette, leaving the inoculated beads free of liquid. The vials were stored at – 80 °C until use as shown in *Figure 11*.

Figure 11: Microbank cryovial used for the storage of bacterial stock culture of B. cereus NVH. ***Source****: Image of MICROBANK ™, ProLab Diagnostics microbial storage vials.*

3.2. Production and Enumeration of *B. cereus* NVH spores

The optimal conditions and ideal growth media for spore production were tested by preparing different batches of spores. Sporulation of *B. cereus* NVH was carried out on liquid Broth (LB) medium, liquid agar (LB), 2x Schaeffer's glucose (2xSG) liquid medium and 2x Schaeffer's glucose agar.

3.2.1. Spore production in liquid broth (LB)

The first batch of spores (A) were produced using the liquid broth. To produce spores in LB medium, an overnight culture was first prepared by inoculating a bead from the Microbank cryovial tube (bacterial stock culture) into a sterile 250 ml Erlenmeyer flask containing 50 ml LB medium. The flask was then incubated at optimal temperature (37°C) with continuous orbital shaking (200 rpm) in a New Brunswick Scientific Innova40 incubator shaker for 18-24 hours. The next day, one drop from the overnight bacterial cell suspension was added into each of two 500 ml Erlenmeyer flask containing 70 ml of LB medium. The flasks were incubated at 37 °C while shaking at 200 rpm. The degree of conversion of vegetative cells to spores was inspected every day using a differential phase contrast microscope (Leica DM2000) at Phase 3 and 1000x magnifications to estimate the approximate ratio of spores to vegetative cells. *Bacillus cereus* spores appeared bright and oval while vegetative cells formed large rods. Mother cells were also observed with oval inclusions indicating the onset of spore formation. After 4 days of incubation, spore production was successful in LB broth with a 90% sporulation rate.

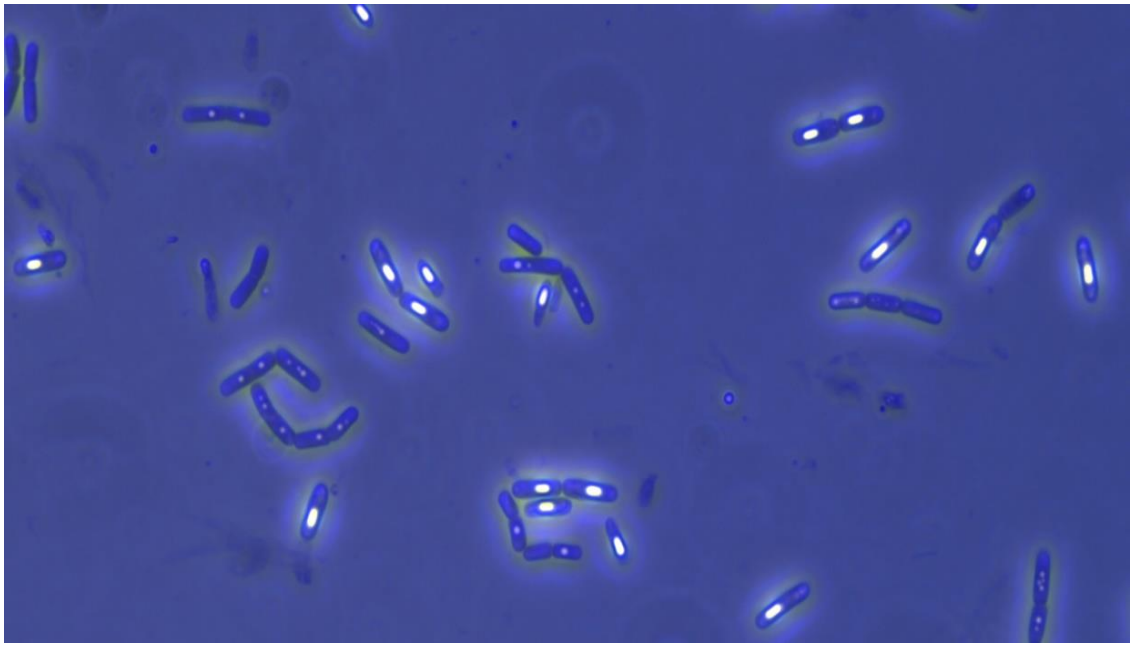


Figure 12: Microscopic images B. cereus NVH *at day 1 sporulation showing mother cells with bright oval inclusion (Scale:25 um)*

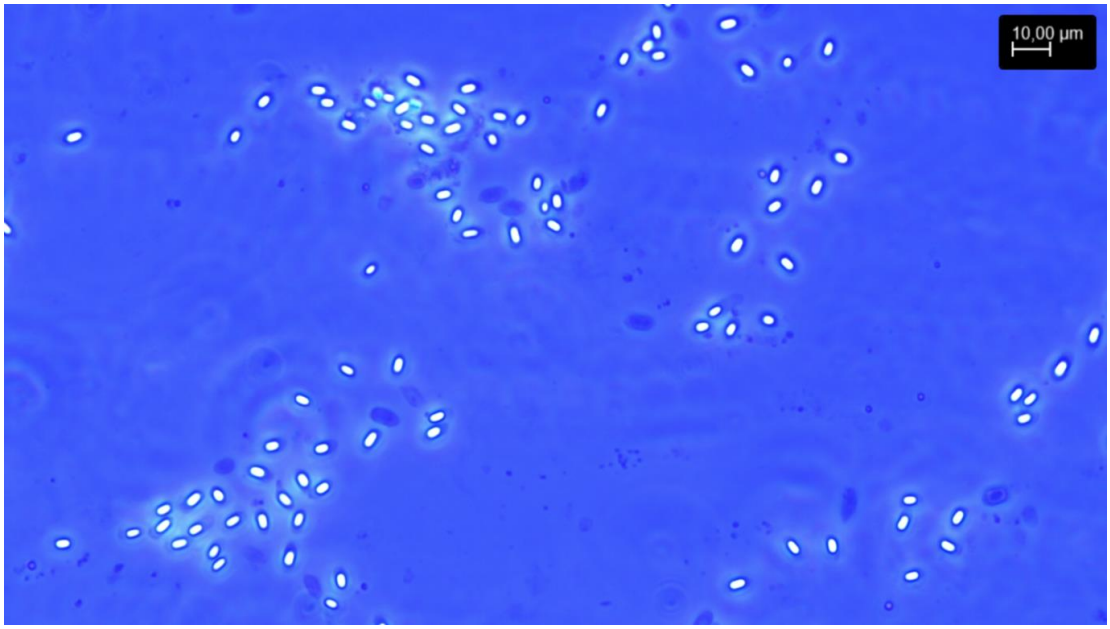
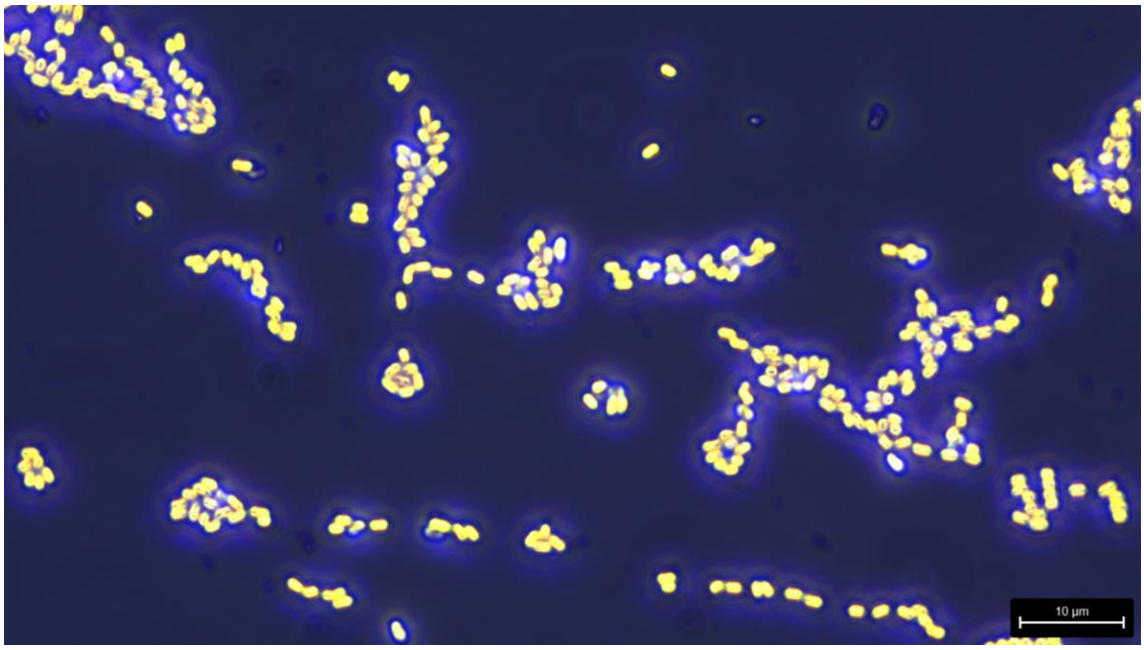


Figure 13: B. cereus NVH *at day 2 sporulation. Spores appear bright and oval. No mother cells observed at this stage.*

(A)



(B)

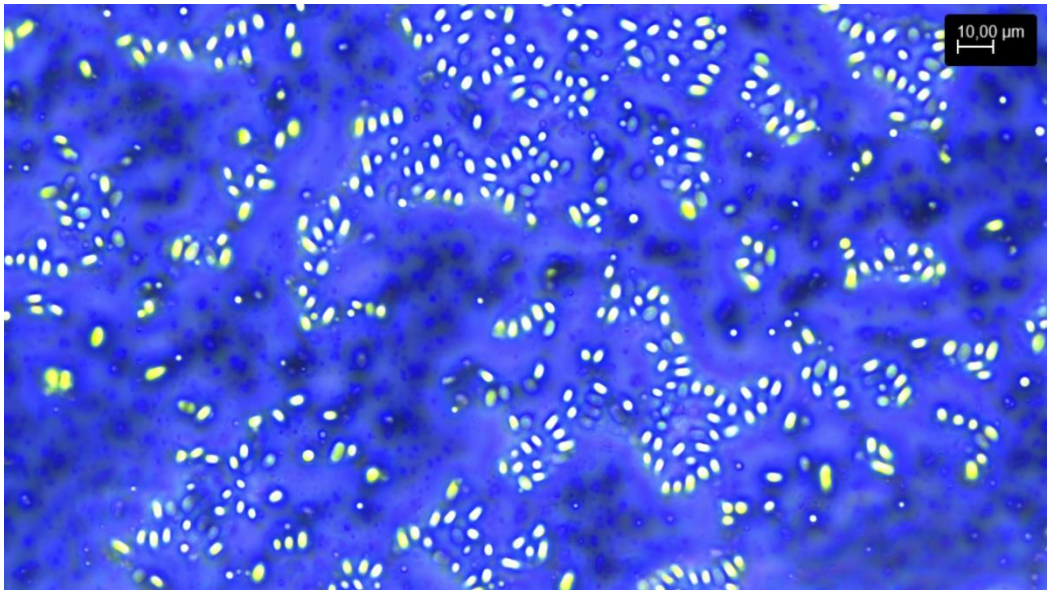


Figure 14: B. cereus NVH at day 3 and 4 of sporulation (A and B) respectively. At this point, the spores are light refracted, dormant and resistant.

3.2.2. Spore production on LB agar

Sporulation on LB agar (B) was performed to assess and compare the quality of sporulation. A surface spread of 0.1 ml of the overnight culture was performed on three LB agar plates using a sterile L-shaped spreader. All plates were incubated at 37 °C. The degree of sporulation and

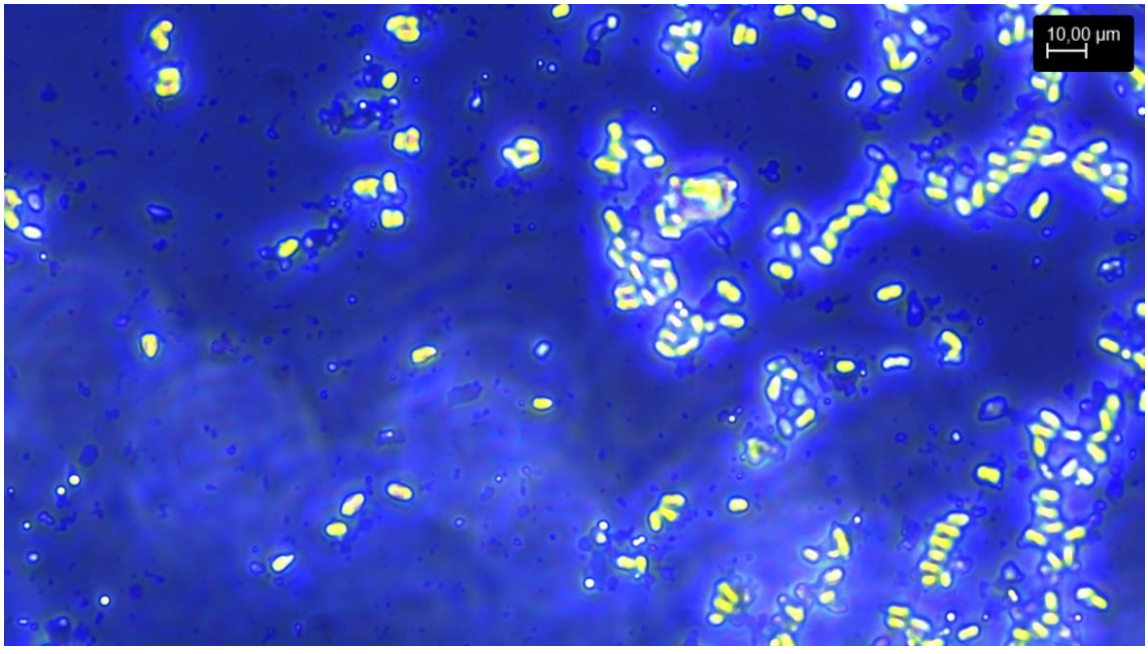
optimal time for harvesting was determined by observing a sample of the spore culture under the microscope each day as described in the microscopic analysis (See 3.3.4). After 4 days, the level of sporulation was more than 90% sporulation.

3.2.3 Harvesting of Spores on LB agar

Under aseptic techniques, the spores were harvested from the agar plates by flooding the surface of the agar plates with 2 ml cold sterile Milli-Q water per plate (total volume of 6 ml), and gently scraping the surface of the plates with a sterile L-shape spreader to dislodge the spores. Spores from each LB agar plate was aspirated and pooled separately in one sterile 50-mL Falcon tube kept on ice.

3.2.4 Washing of *B. cereus* NVH spores

The two tubes containing spores from LB medium and LB agar were washed three times by centrifugation at 10000 x g for 10 minutes at 4 °C on the first day. In each washing step, the supernatant was removed, and the pellet was resuspended in 18 ml (1/4 of total original volume) cold sterile Milli-Q water. The pellets were homogenized by vortexing and then refrigerated for 24 hours at 4 °C while rotating. The next 4 days, the spore suspensions were centrifuged at 20000 x g for 20 minutes at 4 °C and suspended in 15 ml cold sterile Milli-Q water. A whitish fluffy layer of dead mother cells was observed in each tube as part of the spore suspension after centrifugation. The spores were observed under the microscope at 1000x magnification each day. Sporulation on agar plates confirmed the presence of clean, pure spores with some spore clusters while sporulation in the broth medium were single and more widespread. **(A)**



(B)

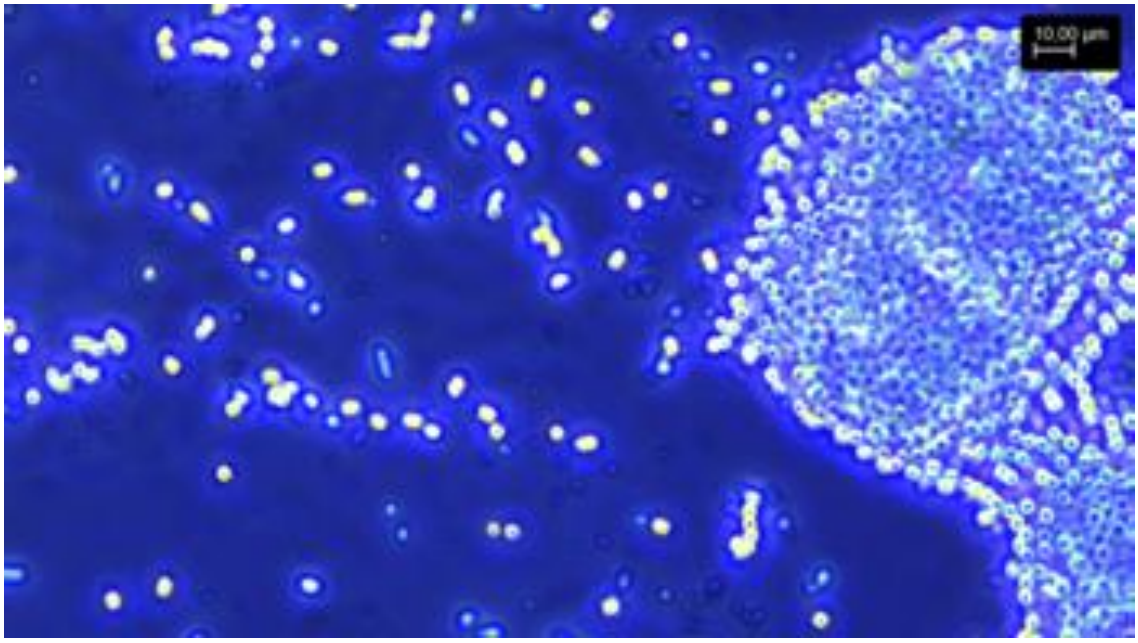


Figure 15: A microscopic image of sporulation on LB agar (image A) and LB medium (image B). Pure spores forming clusters on LB agar while sporulation in LB medium appear more widespread.

3.3 Enumeration of *B. cereus* NVH spores

3.3.1 Spread Plating

The number of spores per ml of bacterial spore batches A and B (*See Table 1*) were determined by spread plating on LB agar. This was done by performing a serial dilution (150 ul spore suspension in 1350 ul diluent) from 10^{-1} to 10^{-7} in microcentrifuge tubes. Each tube dilution was mixed thoroughly to yield a uniform spore suspension. Quantities of 0.1 ml of spore dilutions (10^{-4} to 10^{-7}) were spread on duplicate LB agar plates. The LB plates were then inverted and incubated at 37 °C for 24 hours until visible colonies were formed. After 24 hours, the plates were observed, and the number of colonies obtained on each plate was enumerated. The results were expressed in colony forming units per millilitre (cfu/ml) of bacterial spores.

3.3.2 Pour Plating

In the pour plate method, 1 ml of spore suspension of the appropriate decimal dilution was placed in the centre of a Petri dish using a sterile pipette. Cooled, molten agar was then poured into the Petri dish containing the inoculum. This was followed by gently swirling the Petri dish to attain a uniform mix of the culture and agar medium. The agar plates were left to solidify and incubated at 37 °C for 24 hours. Surface migration of colonies and spreading were also

observed using the pour plate method. To avoid the surface spreading of colonies, some precautionary measures put in place included:

- An overlay of the solidified agar plate with cooled molten agar to delay the migration of colonies
- Setting the incubation temperature at 30 °C instead of 37 °C to prevent an overgrowth and surface spreading.
- Reducing the incubation time to 18-20 hours instead of 24 hours

Similarly, the results were expressed in colony forming units per millilitre (cfu/ml) of bacterial spores after counting the number of colonies on each plate as determined by the equation below.

$$\frac{CFU}{ml} = \frac{number\ of\ colonies}{dilution\ factor \times volume\ plated\ (ml)}$$

3.3.3 Eddy Jet method

The Eddy Jet method was also performed to select the ideal method for enumerating *B. cereus* NVH spores. Spiral plating using Eddy Jet is a widely acceptable technique used in performing viable bacterial counts. First, the appropriate decimal dilutions of the bacterial suspension were prepared using buffered peptone water as diluent in a 1:10 dilution series. The samples were surface plated on LB agar plates by use of a mechanical Spiral Plater (Eddy Jet, IUL Instruments, Barcelona, Spain). Plates were allowed to dry and incubated overnight at 30°C. After incubating the plates, colonies appear distributed along the spiral's track with a radial decrease in concentration. The number of bacterial colonies (cfu/ml) were then enumerated using a specific counting grid, where counting starts in the outermost sectors of the plate and moves inward.

After several counts, the spread plate method was chosen as the ideal method of enumeration as pure and well isolated colonies were observed in the repeated experiment. The spore concentration was estimated at 10^8 spores/ml in both batches of LB medium (A) and LB agar (B) stock solutions.

3.3.4 Microscopic analysis and enumeration of *B. cereus* NVH

A differential phase contrast microscope (Leica DM2000) at Phase 3 and 1000x magnifications was used in observing and enumerating bacterial vegetative cells and spores. For routine microscopic observations, a microscopic slide of the bacterial spore culture is prepared, and a drop oil (oil emersion) is placed at the centre of the covered slide. The slide is then mounted

onto the stage of the phase contrast microscope, focussed, and observed under 1000x magnifications at phase 3. For enumeration of bacterial vegetative cells spores, the Thoma counting chamber was used. The Thoma counting chamber is a special slide with a frame of known surface marked on it, wherein a fixed volume of the cell suspension under study is placed. The frame of the counting chamber contains a large central square (with a 1 mm^2 which can be seen in its entirety with the 10x objective). This large central square is divided into 16 medium squares (with the 40x objective the medium squares can see completely), each with 25 small squares inside (9 of them are divided in half). In enumerating bacterial spores, the spore suspension was placed under the coverslip till a height of 0.1 mm is attained. The microscope was then switched to 40x objective. All the cells in the 16 medium squares were counted and the concentration of the sample was estimated as

N x 10^4 cells/ml

If prior to counting, a concentrated or diluted initial sample was prepared, the concentration or dilution factor (f) was taken into account and estimated as:

N x 10^4 x f cells/ml

3.4 Spore production in 2x Schaeffer's glucose (SG) liquid medium

The next batch of spores (C and D) were prepared using the SG liquid 2x Schaeffer's glucose (SG) liquid medium and SG agar respectively. For sporulation in SG medium (batch C), one drop from the overnight bacterial cell suspension was added into each of three 500 ml Erlenmeyer flasks containing 70 ml of SG liquid medium each. All flasks were incubated at 37 °C while shaking at 200 rpm. The degree of conversion of vegetative cells to spores was inspected every day using a differential phase contrast microscope (Leica DM2000) at Phase 3 and 1000x magnifications to estimate the approximate ratio of spores to vegetative cells. After 3 days of incubation, spore production was successful, with more than 90% rate of sporulation.

3.4.1 Spore production on 2x Schaeffer's glucose (SG) agar

Sporulation on 2x SG agar (B) was performed following a similar process as sporulation on LB agar. Similarly, a surface spread of 0.1 ml of the overnight culture was performed on three 2xSG agar plates using a sterile L-shaped spreader. All plates were incubated at 37 °C. The approximate ratio of spores to vegetative cells and optimal time for harvesting was determined by viewing a sample of the spore culture under the microscope each day. After 3 days, the level of sporulation was more than 90% sporulation. The spores were harvested into one sterile 50-

mL Falcon tubes kept on ice, under aseptic conditions as outlined in the protocol for harvesting spores on LB agar ***(See 3.2.3)***.

The two tubes containing spores from 2x SG medium and 2x SG agar were washed three times by centrifugation at 10000 x g for 10 minutes at 4 °C on the first day and 20000 x g for 20 minutes at 4 °C in the next 4 days. The spores were observed under the microscope at 1000x magnification each day to ascertain the degree of sporulation.

The spread plate method was used in the enumeration of bacterial spores grown on 2x SG medium and SG agar respectively. After several counts, the estimated bacterial number was 10^8 spores/ml in both batches of 2x SG medium (C) and SG agar (D).

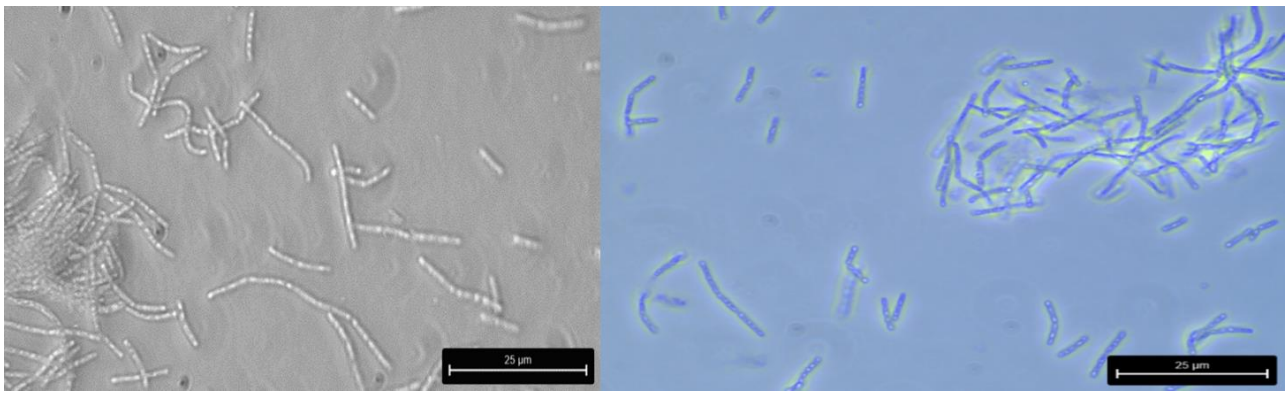


Figure 16: The onset of sporulation on 2x SG agar. Vegetative cells forming large rodlike structures. Mother cells containing oval-shaped inclusions.

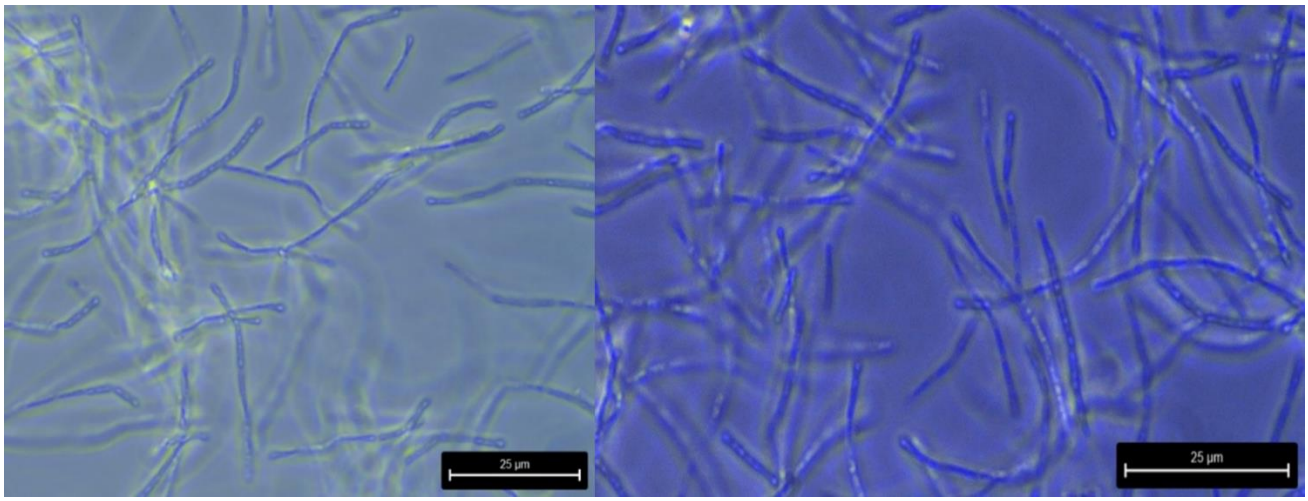


Figure 17: *The start of sporulation in 2x SG medium. Vegetative cells form long rods with bright oval inclusions.*

Table 7: A summary of the spore batches and growth media used in this study.

Spore batch	Growth media	Washing solution	Centrifugation Speed	Observation	Spore Quality
A	LB medium	Milli-Q water	10000xg (1 day), 20000xg	Single spores, clean, pure, mother cells with inclusions, Sporulation after 4 days	90% after 4 days
B	LB agar	Milli-Q water	10000xg (1 day), 20000xg after	pure spores, sporulation after 4 days, aggregates from agar, less streaming compared to broth	90% after 4 days
C	2x SG broth After 18 hrs	Milli-Q water	10000xg (1 day), 20000xg after	Long rodlike filaments with inclusions	20%
	24 hrs			More inclusions, fewer filaments	40%
	48 hrs			spores released, not enough sporulation	60%
	72 hrs			High sporulation	90 %
D	2x SG agar	Milli-Q water	10000xg (1 day), 20000xg after	Short rods with inclusions	90% after 3 days

3.5 Investigating bacterial growth rate using Bioscreen C

The Time to detection experiments (TTD) based on turbidometry using an automated Bioscreen C is a useful method for estimating microbial growth parameters such as lag time and growth rate. In this study, the Bioscreen C (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd, Finland) was used to measure the turbidity (optical density) of the bacterial solution samples in a sterile 10 × 10 (10 columns, 10 rows) wells honeycomb microtiter plates with lid, per test.

3.5.1 Bioscreen C growth test

Prior to the Bioscreen experiment, bacterial cultures of *B. cereus* NVH were grown over-night under growth conditions of (24 h / 37 ºC /200 rpm). Serial dilutions (10^{-1} to 10^{-6}) of the overnight culture were prepared in fresh LB medium. A 200 ml aliquot of each serial dilution were dispensed into the microtiter plate wells (in three replicates). The Bioscreen analyzer was programmed to record the optical density (OD) of each well at 600 nm every 10 min for 72 h at a specified setup temperature program while shaking every 10 s before each reading.

3.5.2 Analysis of data

OD_{600} recordings from the Bioscreen experiment were exported into Excel. The optical density from serial dilutions (10^{-2} to 10^{-6}) were taken and the mean of the five sets of OD data was calculated and used to create growth curves (absorbance plotted against time). From the growth curve, the Time to detection for each serial dilution was related to bacterial number. The TTD equates to the time taken for the OD_{600} to increase to 0.2. A standard calibration curve was obtained for each strain by plotting TTD against the concentration of the initial inoculums (log10 cfu/ml). The slope of the curve was then calculated using standard Microsoft Excel regression analysis.

3.6 Effect of preservatives and pH on growth pattern of *B. cereus* NVH *spores*

The effect of preservatives was investigated on bacterial spores of *B. cereus* NVH using Bioscreen C. Stock solutions of the preservatives NaCl, KCl and $NaNO_2$ as well as different pH values were prepared. The stock solutions were further diluted to obtain the desired concentrations as outlined in Table 8.

Table 8: Concentrations of preservatives used in Bioscreen growth test.

Preservatives	Stock Concentration	Preparation	User Concentrations	Concentration after mixing with bacterial suspension (50:50)
NaCl	8%	16,89 g NaCl in 200 ml LB medium	8%	4%
		25 ml of stock + 25 ml LB	4%	2%
		12 ml of stock + 37.5 ml LB	2%	1%
KCl	8%	16,89 g KCl in 200 ml LB medium	8%	4%
		25 ml of stock + 25 ml LB	4%	2%
		12 ml of stock + 37.5 ml LB	2%	1%
$NaNO_2$	1000 ppm (1mg/ml)	0.2 g $NaNO_2$ in 200 ml LB medium (sterile filtration)	Dilute to 240 ppm	
			(50 ml 240 ppm-solution: 12 ml stock + 38 ml LB)	240 ppm
			Dilute to 120 ppm	120 ppm
			(50 ml 120 ppm-solution: 6 ml stock + 42 ml LB)	
			Dilute to 60 ppm	60 ppm
			(50 ml 60 ppm-solution: 3 ml stock + 47 ml LB)	
pH	3.0			4.0

	4.0			5.0
	5.0			6.0
	6.0			7.0

3.6.1 Bioscreen C growth experiment using preservatives

As confirmed, the initial concentration of bacterial spores in spore batch B (LB broth) was 10^8 cfu/ml, and this was further diluted in LB medium to a final concentration of 10^4 cfu/ml. The inoculum level was confirmed by a plate count on LB agar and direct microscopic counts using the Thoma counting chamber. After, confirming the final concentration, a 50:50 sample concentration (100 µl from each concentration of preservatives and pH, and 100 µl from bacterial suspension) were dispensed into the microtiter plate wells (in three replicates) to attain a final volume of 200 µl/well. A control experiment which comprised of bacterial sample concentration of 10^4 cfu/ml and a blank, composed of pure LB medium (without bacteria) were also setup.

Table 9: Experimental design for the Bioscreen C mircotitre plate setup using preservatives

	01	***11***	***21***	***31***	***41***	***51***	***61***	***71***	***81***
1	4 % NaCl	4 % NaCl	4 % NaCl	2 % NaCl	2 % NaCl	2 % NaCl	1 % NaCl	1 % NaCl	1 % NaCl
2	4 % KCl	4 % KCl	4 % KCl	2 % KCl	2 % KCl	2 % KCl	1 % KCl	1 % KCl	1 % KCl
3	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$	1000 ppm $NaNO_2$
4	pH 4	pH 4	pH 4	pH 5	pH 5	pH 5	pH 6	pH 6	pH 6
	pH 7	pH 7	pH 7						
5	***LB***	***LB***	***LB***	***LB***	***LB***	***LB***	***LB***	***LB***	***LB***
6	***Control***	***Control***	***Control***	***Control***	***Control***	***Control***	***Control***	***Control***	***Control***

The growth rate was followed for 4 days by monitoring the increase in absorbance at 600 nm (OD_{600}) in a BioScreen C microplate reader. BioScreen monitored the growth of bacteria by reading OD_{600} every 10 min for 4 days at a specified setup temperature program while shaking every 10 s before each reading. The experiment was repeated to confirm and compare results obtained from the experiment.

3.6.2 Data Analysis

OD_{600} recordings from the Bioscreen experiment were exported into Excel. The OD values from Bioscreen were analyzed and the averages of the six replicates (three replicates in two separate experiments) were calculated for each type of preservative in various concentrations. The averages were used to generate growth curves by plotting the OD 600 nm of the cultures versus incubation time. Growth curves of the replicates were also plotted to visualise any changes in variance. The Time to detection was also determined by equating the time taken for the OD_{600} to increase to 0.2.

3.6.3 Confirmatory Test of *B. cereus* NVH growth at pH 4

A test to confirm the growth of *B. cereus* NVH vegetative cells at pH 4 was carried out. First, an overnight culture was prepared and diluted in LB medium to a final concentration of 10^4 cfu/ml. A volume of 20 ml of LB medium was adjusted to pH 3 and used as the preservative in this test. A 50:50 sample concentration (100 µl from each concentration of pH 3, and 100 µl from bacterial suspension) were dispensed into the microtiter plate wells (in three replicates) to attain a final volume of 200 µl/well. A control sample (without bacteria) containing 100ul of LB medium and 100ul of LB at pH 3 was also set up. The experiment was allowed to run for 3 days using the Bioscreen C machine at a specified time and temperature program. The data obtained in this experiment were processed to obtain the growth curves.

3.7 Effect of combined preservatives and pH on *B. cereus* NVH spore growth

The combined effect of preservatives and pH on *B. cereus* NVH spores using Bioscreen C was investigated. Stock solutions of the preservatives NaCl, KCl and $NaNO_2$ as well as different pH values were prepared and combined in different concentrations as defined in Table 10. Similarly, the initial concentration of bacterial spores in spore batch C (LB broth) was 10^8 cfu/ml, and this was further diluted in LB medium to a final concentration of 10^4 cfu/ml. The inoculum level was confirmed by a plate count on LB agar and direct microscopic counts using the Thoma counting chamber. After, confirming the final concentration, a 50:50 sample concentration (100 µl) from each concentration of preservatives and pH, and 100 µl from bacterial suspension) were dispensed into the microtiter plate wells (in three replicates) to attain

a final volume of 200 µl/well. A control experiment which comprised of bacterial sample concentration of 10^4 cfu/ml and a blank, composed of pure LB medium (without bacteria) were also setup. The growth rate was followed for 4 days by monitoring the increase in absorbance at 600 nm (OD_{600}) in a BioScreen C microplate reader. BioScreen monitored the growth of bacteria by reading OD_{600} every 10 min for 4 days at a specified setup temperature program while shaking every 10 s before each reading. The experiment was repeated to confirm and compare results obtained from the experiment.

***Table 10**: Experimental design for the Bioscreen C mircotitre plate setup using a combination of preservatives*

Solution	**Concentrations**	**Final concentrations**
NaCl, pH 3	• 8% NaCl-LB, pH 3 • 4% NaCl-LB, pH 3 (1 ml 8% NaCl-LB, pH 3 + 1 ml LB, pH 3) → 2 ml (1.25 ml) • 2% NaCl, pH 3 (0.75 ml 4% NaCl-LB, pH 3 + 0.75 ml LB, pH 3) → 1.5 ml	• 4% NaCl-LB, pH 4 • 2% NaCl-LB, pH 4 • 1% NaCl, pH 4
NaCl, pH 4	• 8% NaCl-LB, pH 4 • 4% NaCl-LB, pH 4 (1 ml 8% NaCl-LB, pH 4 + 1 ml LB, pH 4) → 2 ml (1.25 ml) • 2% NaCl, pH 4 (0.75 ml 4% NaCl-LB, pH 4 + 0.75 ml LB, pH 4) → 1.5 ml	• 4% NaCl-LB, pH 5 • 2% NaCl-LB, pH 5 • 1% NaCl, pH 5
NaCl, pH 5	• 8% NaCl-LB, pH 5 • 4% NaCl-LB, pH 5 (1 ml 8% NaCl-LB, pH 5 + 1 ml LB, pH 5) → 2 ml (1.25 ml) • 2% NaCl, pH 5 (0.75 ml 4% NaCl-LB, pH 5 + 0.75 ml LB, pH 5) → 1.5 ml	• 4% NaCl-LB, pH 6.2 • 2% NaCl-LB, pH 6.2 • 1% NaCl, pH 6.2
NaCl-NaNO2, pH 3	• 8% NaCl-1000ppm NaNO2-LB, pH 3 • 4% NaCl-500ppm NaNO2-LB, pH 3 (1 ml 8% NaCl-1000ppm NaNO2-LB, pH 3 + 1 ml LB, pH 3) → 2 ml (1.25 ml) • 2% NaCl-125, pH 3 (0.75 ml 4% NaCl-1000ppm NaNO2-LB, pH 3 + 0.75 ml LB, pH 3) → 1.5 ml	• 4% NaCl-500ppm NaNO2-LB, pH 4

		• 2% NaCl-250ppm NaNO2-LB, pH 4 • 1% NaCl-62.5ppm NaNO2, pH 4
NaCl-NaNO2, pH 4	• 8% NaCl-1000ppm NaNO2-LB, pH 4 • 4% NaCl-500ppm NaNO2-LB, pH 4 (1 ml 8% NaCl-1000ppm NaNO2-LB, pH 4 + 1 ml LB, pH 4) → 2 ml (1.25 ml) 2% NaCl-125, pH 4 (0.75 ml 4% NaCl-1000ppm NaNO2-LB, pH 4 + 0.75 ml LB, pH 4) → 1.5 ml	• 4% NaCl-500ppm NaNO2-LB, pH 5 • 2% NaCl-250ppm NaNO2-LB, pH 5 1% NaCl-62.5ppm NaNO2, pH 5
NaCl-NaNO2, pH 5	• 8% NaCl-1000ppm NaNO2-LB, pH 5 • 4% NaCl-500ppm NaNO2-LB, pH 5 (1 ml 8% NaCl-1000ppm NaNO2-LB, pH 5 + 1 ml LB, pH 5) → 2 ml (1.25 ml) 2% NaCl-125, pH 5 (0.75 ml 4% NaCl-1000ppm NaNO2-LB, pH 5 + 0.75 ml LB, pH 5) → 1.5 ml	• 4% NaCl-500ppm NaNO2-LB, pH 6.2 • 2% NaCl-250ppm NaNO2-LB, pH 6.2 • 1% NaCl-62.5ppm NaNO2, pH 6.2

3.8 Heat treatment of *B. cereus* NVH spores

The heat experiment was carried out to assess the level of heat resistance of *B. cereus* NVH spores by a reduction in log numbers at different time and temperature programs.

3.8.1 The Thermal Treatment Process

The bacterial spore suspension in batch C (*See*
Table 7) was used in this experiment. The spore suspension was further diluted one log unit down to obtain a starting number of 10^7. All tubes were kept on ice prior to inactivation to prevent spore germination. Three replicates for each time and temperature program were setup for each experiment. First, a volume of 500 ul of the diluted spore suspension was carefully

pipetted into each of the three Eppendorf tubes (1.5 ml tubes) to prevent spore droplets on the walls of the tubes. The tubes were placed on a floating element and put into a closed water bath. A thermometer was also used to confirm the temperature of the water bath. A non-treated sample was also setup as control. Heat treatments were carried out using different time and temperature programs in a water bath, according to the setup in Table 11.

Figure 18: An image of a floating element with tubes containing the bacterial spore suspension fully immersed in a water bath, set at 95℃ for the heat treatment experiment. (Picture: Nofima Food Microbiology Lab).

Table 11: Time and Temperature program for Heat Treatment of B. cereus* NVH *spores

EXPERIMENT	TIME/TEMPERATURE PROGRAM	COMMENTS
Experiment 1	Control (untreated)	
	70-5min	No significant difference in log reductions between 70℃ and 80 ℃ at 5 and 10 mins
	70-10 min	
	80-5 min	
	80-10 min	
	95-5 min	About 2.5-3.0 log reductions compared to control
	95-10 min	
Experiment 2	Control	

	80-5 min	No significant difference in log reductions at 80℃ at 5 and 10 mins
	80-10 min	
	90-5min	
	90-10 min	About 2 log reduction observed at 90℃ for 10 mins
	95-5 min	
	95-10 min	About 3 log reductions observed at 95℃ for 10 min. *NVH 1230-88* could be quite heat resistance. High log reductions observed at higher temperature and time. Propose: Test strain at temperatures higher than 95℃ in Experiment 3.
Experiment 3	Control	
	95- 5min	
	95-10 min	About 3 to 4 log reductions achieved at 95℃ and 97 ℃.
	97-5 min	
	97-10 min	

After heating, the tubes were removed, and cooled in ice slurry immediately to avoid further inactivation. A volume of 100 ul of each heat treated sample tube was serially diluted and the appropriate serial dilutions were plated on LB agar using the Eddy Jet and Spread plate technique. Heat resistance was determined and compared by counting the number of colonies after 18-20 h incubation at 30°C.

3.9 Inactivation of B. cereus NVH spores in food matrices by heat treatment and high-pressure processing

The inactivation of *B. cereus* NVH spores by thermal treatment coupled with high-pressure processing was investigated in minced meat.

3.9.1 Production of *B. cereus* NVH spores

In this experiment, higher concentration of bacterial spores was needed. To obtain a spore concentration of 10^8 cfu/ml, sporulation was carried out in seven flasks containing 2x SG medium. First, an overnight culture was prepared, and one drop of bacterial spore suspension was added to each flask according to standard protocols. The washing of the bacterial spores was performed with Milli-Q water as described previously (See *Table 7*). After washing, the spore suspensions were up concentrated to achieve a final spore concentration of 10^8 cfu/ml. The new batch of washed spores was labelled as *batch E.*

3.9.2 Inactivation of spores by heat treatment in minced meat

The minced meat was purchased as a commercial product, and its composition is shown in Table 12. Heating was done at 97 °C for 10 minutes.

Table 12: Composition of minced meat used for heating and pressure processing experiments.

Compound	**Protein**	**Fat**	**Salt**	**Energy**	**Carbohydrates**
Amount/100 g	20	14	0.1	205 kcal	0

3.9.3 Food packaging and inoculation

Minced meat was portioned in samples of 100 g and vacuum packed. The samples were then stored frozen at -20 °C. The minced meat was thawed overnight in the refrigerator at 4 °C before use. Then 1.2 ml of the spore suspension was mixed with 20 ml of cold Milli-Q water in a Falcon tube and added to 100 g of minced meat. The sample was mixed thoroughly by using the fingers to achieve a homogenous mixture. Then 6 g out of the 100 g of minced meat containing spores was transferred to plastic bags and sealed as a squared package (5×6 cm). In all, 10 packages of 6g of minced meat were prepared for the pressure and thermal treatments as follows.

- 6 packages for HPP (3 packs each for 40 and 55°C pretreatment)
- 3 packages for thermal treatment at 97°C for 10 min
- 1 package for control (non-treated) sample

To ensure consistent heat distribution throughout the product, minced meat was rolled flat inside the plastic bags as showed in Figure 19.

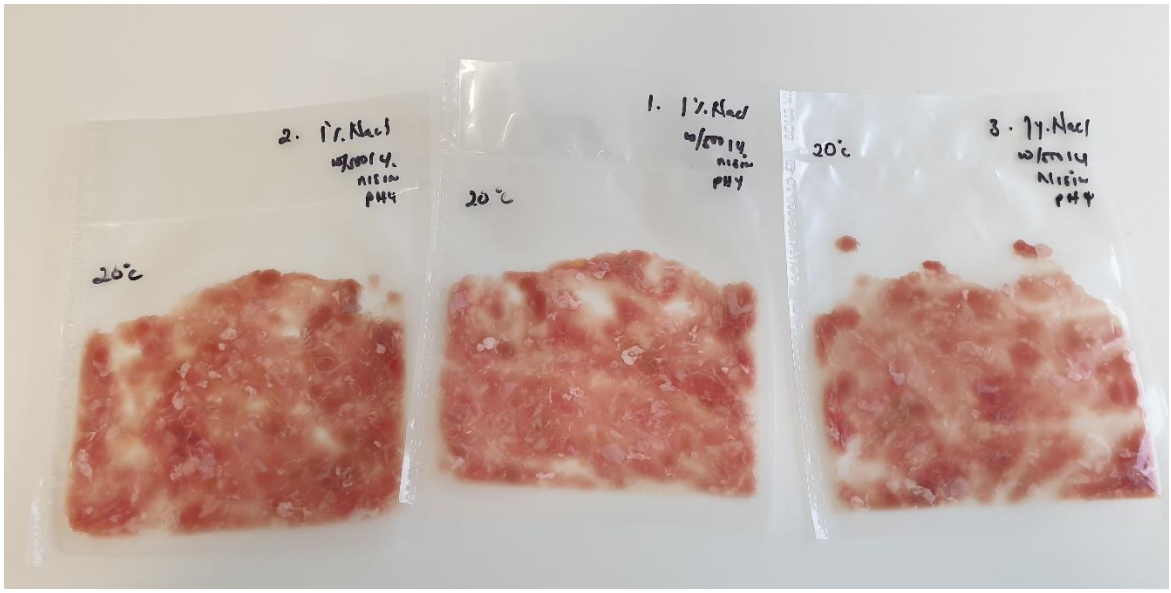


Figure 19: Flattened and sealed plastic bags containing 6g of treated meat sample rolled flat for even heat distribution.

3.9.4 Heating process

The experiment was performed for a temperature-time combination of 97℃ for 10 minutes. Each experiment consisted of three replicates each at the specified temperature/time combination. Non-heat-treated samples with spores were analysed as control samples.

For the heating processes, three parallel samples were immersed in the water bath simultaneously. Plastic bags were attached to a metal rack to prevent them from floating. Each bag was maintained separately from the others in the water bath, to allow water to circulate freely between bags and to achieve a uniform heat distribution in all bags. To minimize a high temperature deviation, the metal rack without the samples was prewarmed in the water bath before starting the experiment. At the end of heat treatment, samples were placed immediately in ice slurry to prevent further inactivation.

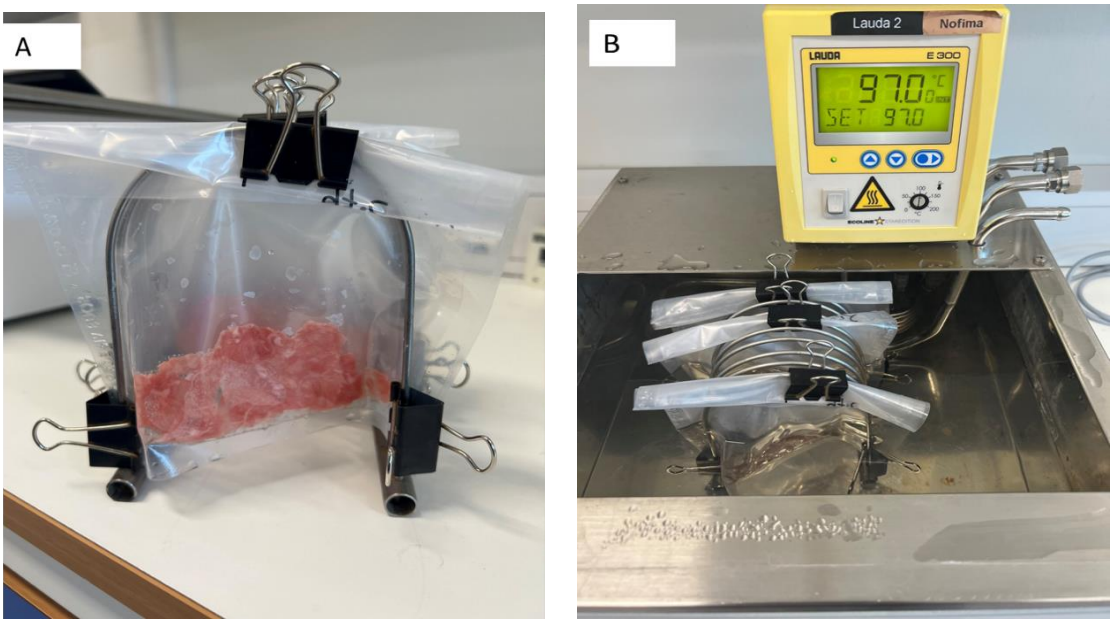


Figure 20: An illustration of thermal inactivation of spores in minced meat sample. (A) 6 g of minced meat containing spores packaged, sealed, and attached to a metal rack for the heating process. (B) heat treatment of minced meat containing spores in water bath at 97℃.

To analyse the concentration of surviving spores after the heat treatment, 5g of the heated sample were combined with 1% peptone water to achieve a total weight of 50 g (1:10) in a stomacher bag for 2 minutes using the Stomacher 41 machine. Initially, the spore suspension had a concentration of 10^8 cfu/ml and was diluted 10-fold in cold Milli-Q water to 10^7. Therefore, the spore concentration in the stomach bag was 10^6 cfu/ml due to the further dilution with the 1% peptone water (1:10). After, 10 ml of the homogenized sample were transferred from stomacher bag into a 15 ml sterilized Falcon tube. Further 10-fold dilutions were made using sterile Milli-Q water. In the next step, Eddy Jet method was used where a volume of 49.2 ul was spirally spread on LB agar plates. This was allowed to dry for some minutes and followed by incubation at 30 °C. After 24 hours the number of colonies was counted, and the log reductions were determined.

3.10 Inactivation of spores by pressure processing in minced meat

The effect of high pressure (600 MPa) for 2 min at two different temperatures (40 and 55 °C) on *B. cereus* NVH spores was investigated.

3.10.1 Food inoculation and packaging

Sample preparation steps were the same as in the previous experiment (section 3.8.2). The inoculated meat samples were packed in 5×6 cm plastic bags as described before to allow for rapid heat and pressure transmission.

3.10.2 High pressure processing

A high hydrostatic pressure machine QFP 2L-700 (Avure Technologies Inc., Columbus, USA) was used for pressure and pressure combined heat treatment of *B. cereus* spores. Prior to the combined pressure–heat treatment applications, plastic bags containing minced meat were preheated in a water bath at desired temperatures (40 and 55 ℃) for 2 minutes to achieve the target temperature. Two different HPP treatments were employed to evaluate the inactivation of spores. All samples were treated at pressure of 600 MPa at 40 and 55 °C and pressure holding times of 2 minutes.

After processing, treated samples were immediately immersed in ice slurry prior to enumeration of surviving spores. Samples were serially diluted in sterile Milli-Q water, plated on LB agar (Eddy Jet method) and incubated at 30 °C for 18 to 20 h. All samples were analysed in 3 replicates and average counts were obtained. In addition, three non-treated samples were analysed as control in each experiment to determine the initial number of spores. Table 13 shows a summary of the pressure and temperature used during the HPP process cycle in this study.

Table 13: A summary of applied condition for pressure and pressure-thermal treatment of B. cereus spores.

Preheating Temperature/time	**Pressure Temperature**	**Pressure holding time**	**Number of Samples**
(control)	-	-	1
40 °C /2 minutes	600 MPa /40 °C	2 minutes	3
55 °C /2 minutes	600 MPa /55 °C	2 minutes	3

3.11 Test of *B. cereus* NVH growth at 8℃ and 15℃ with combined preservatives

The vegetative cell growth of *B. cereus* NVH was tested at 8℃ and 15℃ with a combination of preservatives (NaCl adjusted at pH 4) using Bioscreen C. In this experiment, an overnight culture was prepared and diluted to 10^4 cfu/ml. Dilution series from 10^4 cfu/ml were prepared (10^{-1} and 10^{-2}) and plated on LB agar to enumerate bacteria cell numbers.

Specified volumes of preservatives were combined as outlined below. A 50:50 sample concentration (100 µl from each concentration of preservatives and 100 µl from bacterial suspension) were dispensed into the microtiter plate wells (in six replicates) to attain a final volume of 200 µl/well. A control experiment which comprised of bacterial sample concentration of 10^4 cfu/ml and a blank, composed of pure LB medium (without bacteria) were also setup. Each sample well was carefully sucked up and down into the Finn-pipette to homogenise the liquid. In all, 10 sets (8ºC: day 0, 7, 14, 21; (11ºC: day 0, 7, 14, 21) of each preservative and bacterial suspension were prepared. After, the OD of the two sets at day 0 (first day) was measured using Bioscreen. The remaining of the two sets (day 7 to 21 of each set) were then subjected to temperatures 8℃ (first set) and 15℃ (second set) under controlled conditions to minimize temperature losses. Measurements were carried out again at regular intervals every 7 days for 21 days and determine OD at day 21. Data obtained from each period were collected and used to obtain growth curves.

Table 14: Concentrations of Preservatives

Initial Concentration of preservatives	Final concentration of preservatives
2% NaCl, no pH adjustments	1% NaCl, no pH adjustments
2% NaCl, pH 3	1% NaCl, pH 4
2% NaCl, pH 4	1% NaCl, pH 5
LB medium	LB medium
LB+Bacteria	LB+Bacteria

3.12 The combined effect of selected preservatives (Nisin and NaCl at pH) on *B. cereus* spore growth using Bioscreen

The effect of Nisin and NaCl at pH 4 on the growth of *B. cereus* NVH vegetative cells was studied using Bioscreen. First, an overnight culture was prepared and then diluted to a final concentration of 10^4 cfu/ml. A stock solution of nisin was also prepared in accordance with the EFSA (European Food Safety Authority) panel. According to this protocol, 1 IU would correspond to 0.025 µg of nisin and therefore µ1 g of nisin is equivalent to 40 IU (Maged Younes et al., 2017). In this study, nisin was used in its active form, 2.5% (wt/wt) (Sigma) and

a stock solution of nisin containing 100,000 IU/ml, equivalent to 100 IU/ul pure nisin was prepared, by dissolving 0.5 g of nisin in 0.02 M HCl (0.5 ml) and adding 4.5 ml of distilled water in accordance with a protocol by Lee et al., (2015). (Lee et al., 2015; Prado-Acosta et al., 2010). A volume of 20 ml of 2% NaCl solution adjusted at pH 3 was also prepared separately. The stock nisin solution was further diluted (5 and 8 times) using the 2% NaCl solution at pH 3 in two separate flasks to obtain two different solutions of concentrations 1000IU/ml and 200 IU/ml respectively as detailed in the following protocol.

1. A stock solution of 2% NaCl solution was made by dissolving 1.689 g NaCl in 20 ml LB medium (20ml of 2% NaCl)
2. Respective dilutions of nisin solution were prepared using the 2% NaCl (instead of dH_2O)

   Stock 1: 1 ml of stock nisin in 4 ml of (20 ml 2% NaCl) = 1000 IU/ml=20 IU/ul (x5)

   Stock 2: 1 ml of stock nisin in 7 ml of (20 ml 2% NaCl) = 200 IU/ml=12.5 IU/ul
3. Adjust solutions to pH 3

The contents of Stock 1 and 2 are

Stock 1: 2% NaCl, 1000 IU/ml nisin, pH 3

Stock 2: 2% NaCl, 200 IU/ml nisin, pH 3

After, a 50:50 sample concentration (100 µl from Stock preparations and 100 µl from bacterial suspension) were dispensed into the microtiter plate wells (in three replicates) to attain a final volume of 200 µl/well. Controls of stock 1 and stock 2 contained all preservatives and nisin without bacteria at final concentrations of 1% NaCl, 500 IU/ml nisin, pH 4 and 1% NaCl, 100 IU/ml nisin, pH 4 respectively. The Bioscreen was programmed to monitor the growth of bacteria at a specified temperature of 37°C, expressed as increase in absorbance (OD_{600}), every 10 min for 3 days and with shaking of the microtiter tray 10 s before each reading.

3.12.1 Data Analysis

OD_{600} recordings from the Bioscreen experiment were exported into Excel. The OD values from Bioscreen were analyzed and the averages of the three replicates were calculated for each well in various concentrations. The averages were used to generate growth curves by plotting the OD 600 nm of the cultures versus incubation time. Growth curves of the replicates were also plotted to visualise any changes in variance. The Time to detection was also determined at 0.2.

Table 15: Stock solutions of preservatives and nisin used in Bioscreen growth test.

1	2	3	4	5	6	7	8	9	Control 1	Control 2
50:50 of Stock 1 A+bac									Stock 1	LB+bac
50:50 of Stock 2 + bac									Stock 2	LB+bac

3.13 The combined effect of preservatives and HPP on *B. cereus* spore growth

The combined effect of preservatives and HPP on spore inactivation by HPP were investigated. In this experiment, nisin at concentrations of 100IU and 500 IU were combined with 1% NaCl at pH 4 and subjected to pressure-thermal treatments of HPP at 55°C.

3.13.1 Food packaging and inoculation

Vacuum packed minced meat weighing 100g each were portioned based on two main preparation methods namely: pH adjusted samples and non-pH adjusted samples. Nisin solution was also prepared by dissolving 0.5g of 2.5% active Nisin in 500ul of 0.02M HCl. A volume of 4.5 ml of distilled water was then added and mixed thoroughly to obtain s stock nisin solution. The protocol for the sample preparations is outlined as follows.

3.13.2 pH adjusted samples

1% NaCl solution was prepared by mixing 2.4 g NaCl and 40 ml of distilled H_2O (1% NaCl) set at pH 4. The solution was then added to 200g meat.

2.4 ml of concentrated spore suspension (10^7) was measured and dripped on the meat sample and mixed thoroughly with the fingers. The sample was then packaged as follows.

- 3 bags of the meat sample weighing 6g each (1% NaCl, pH 4)
- 2500 ul nisin solution added to 60 g of the meat sample and packed into 6 bags (6 g each) (1% NaCl w/500 IU nisin, pH 4). Out of the 6 bags, 3 bags were subjected at HPP at room temperature (20°C) and HPP at 55°C were applied to the remaining 3 bags.

3.13.3 Non-pH adjusted samples

1% NaCl solution was prepared by mixing 2.4 g NaCl and 40 ml of distilled H_2O (1% NaCl) with no pH adjustments. The solution was then added to 200g meat.

2.4 ml of concentrated spore suspension (10^7) was measured and dripped on the meat sample and mixed thoroughly with the fingers. The sample was then packaged as follows.

- 3 bags of the meat sample weighing 6g each (1% NaCl)
- 3 bags of the meat sample weighing 6g each (1% NaCl) was used as control with no HPP applied.
- 250 ul of nisin solution mixed with 30g of the meat sample and packed into 3 bags of 6g each (1% NaCl, w/100IU nisin)
- 1250 ul of nisin solution mixed with 30g of the meat sample and packed into 3 bags of 6g each (1% NaCl, w/500IU nisin)

All plastic bags were labelled accordingly and sealed as a squared package (5×6 cm). To ensure consistent heat distribution throughout the product, minced meat was rolled flat inside the plastic bags as described in Figure 19.

The meat sample packages each containing 6 g of inoculated minced meat (with combined preservatives) were then subjected to a pressure of 600 MPa after preheating at 55 °C for a holding time of 2 min. Each treatment was performed in triplicate and the experiment was repeated twice. The average log reductions of in both experiments were used to obtain a graph where spore log reductions (log N_0/N) were plotted against treatment conditions.

Figure 21: Image of HPP machine showing the loading chamber where sample packages are placed. Picture: NOFIMA Food Processing Laboratory.

4. RESULTS AND DISCUSSION

Several experiments with *B. cereus* vegetative cells and spores have been carried out in this thesis.

4.1. Bioscreen C experiments

In this experiment, the effect of initial bacterial number on growth of *B. cereus* NVH vegetative cells was investigated.

4.1.1 Investigating the effect of initial bacterial number on growth rate

Turbidity data were obtained from Bioscreen C, by which absorbance readings at 600 nm were measured every 10 minutes. Growth curves for each dilution was plotted.

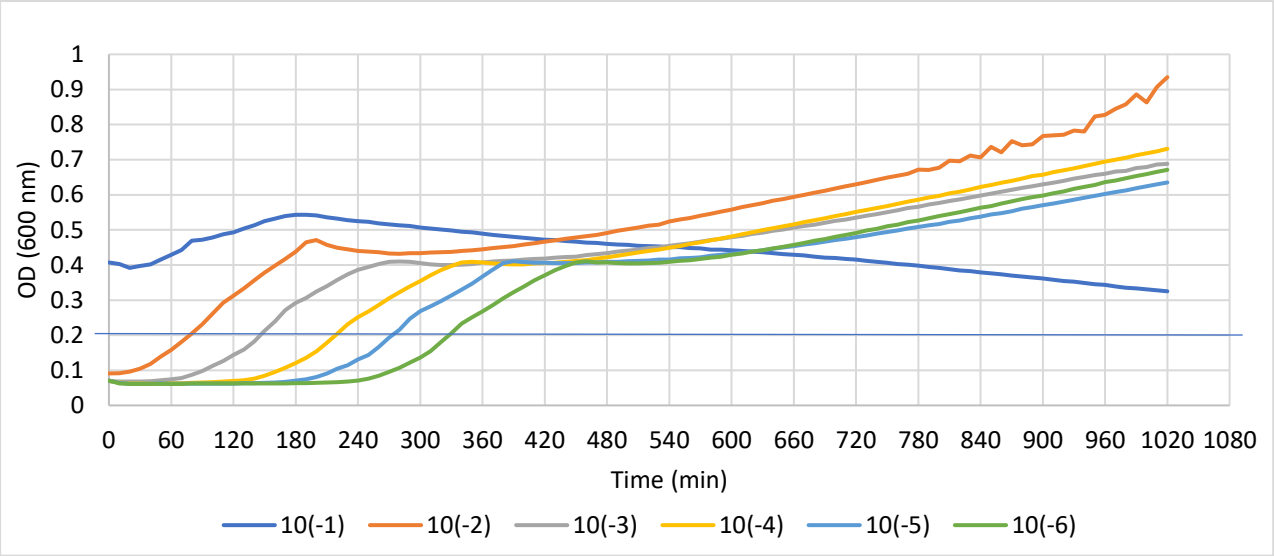


Figure 22: Turbidity growth curves of B. cereus NVH *in LB medium at 37 °C, obtained at OD (600 nm) at 0.2. The Time to Detection (TTD) is recorded for each of 6 decimal dilutions ranging from -1 to -6, in decreasing order of concentration.*

According to *Figure 22*, it can be deduced that the lower the dilution series (10^{-1}), the higher the cell number and the faster the time taken to reach detection (OD at 0.2). For instance, the TTD for -2 decimal dilution was observed at 80 mins at OD of 0.2 while at the same optical density, it took 330 mins for the most diluted suspension (-6) to reach detection. The OD of the lowest decimal dilution (-1) was observed at 0.4 and this could be attributed to the high bacterial concentration or cell numbers which could not be detected by the Bioscreen C. The TTD of each decimal dilution was then used to create a calibration curve as illustrated in Figure 23. From the results of the calibration curve, a linear regression was observed at R^2=0.99, which indicates a good correlation between the OD data and cell numbers. This initial growth experiment showed that the strain grew well in LB and the Bioscreen machine functions well.

It also shows a similar growth curve independent of the initial numbers and there was a linear relationship between dilutions and TTD.

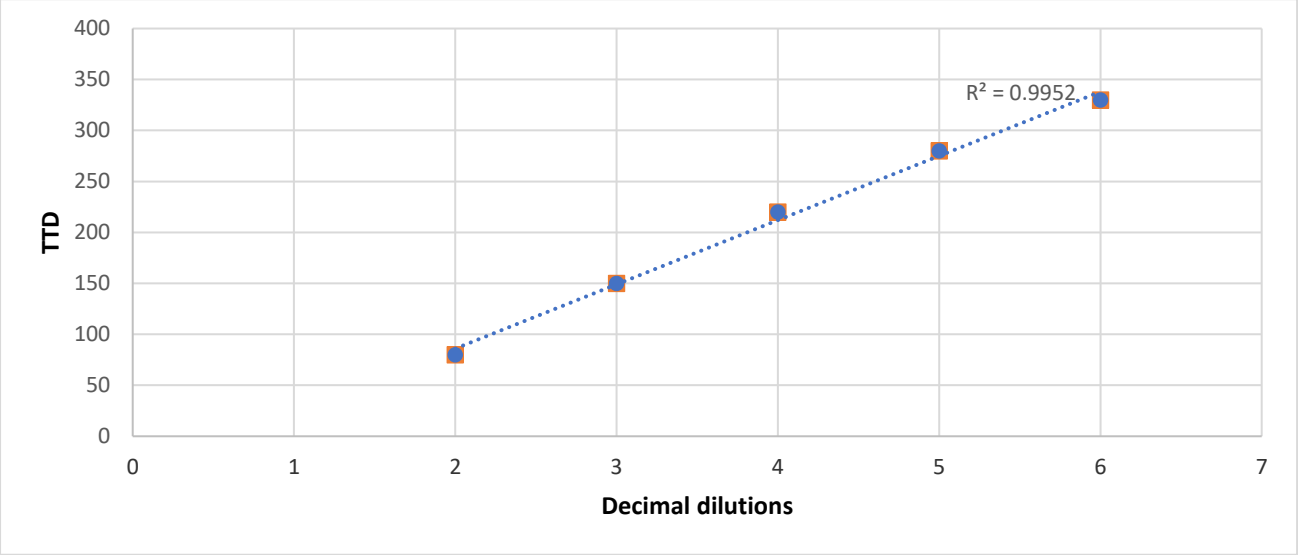


Figure 23: Calibration curve for B. cereus NVH *in LB medium at: TTD (detection times) plotted against the decimal dilutions of B. cereus.*

4.2 Effect of preservatives and pH on growth pattern

Factors such as temperature, pH, salinity, atmosphere, presence of additives can affect the growth of *B. cereus* NVH in foods. In this study Bioscreen C was used to study the growth of *B. cereus* NVH spores in the presence of different concentrations of preservatives. Table 16 describe the time to detection of each preservative at different concentration in two replica experiments.

Table 16: Time to detection of the different preservatives at different concentrations used in experiment A.

Preservative	Concentration (%)	TTD (min)	Max OD	Time to max OD
NaCl	4%	1740-830	0.25-2.23	2100-1210
	2%	680-550	0.9-0.38	1800-1220
	1%	370-380	0.9-0.28	1700-690
KCl	4%	610-650	0.3-0.6	1200-1210
	2%	490-500	0.4-0.4	1200-1216
	1%	260-290	0.6-0.3	1190-1210

$NaNO_2$	4%	330-260	1.03-0.5	2030-1220
	2%	310-240	0.93-0.6	1980-1210
	1%	310-220	1.18-0.67	2010-1210
pH 4		2310-ng	n.g	n.g
pH 5		n.g-1150	0.07-0.2	n.g-1300
pH 6		1750-1010	n.g	n.g
pH 7		480-290	1.12-0.6	2170-1230
LB		280-310	0.32-0.32	610-620

***Where no growth was observed, 'n.g' was used to represent this.*

4.2.1 Effect of NaCl on *B. cereus* NVH spore growth

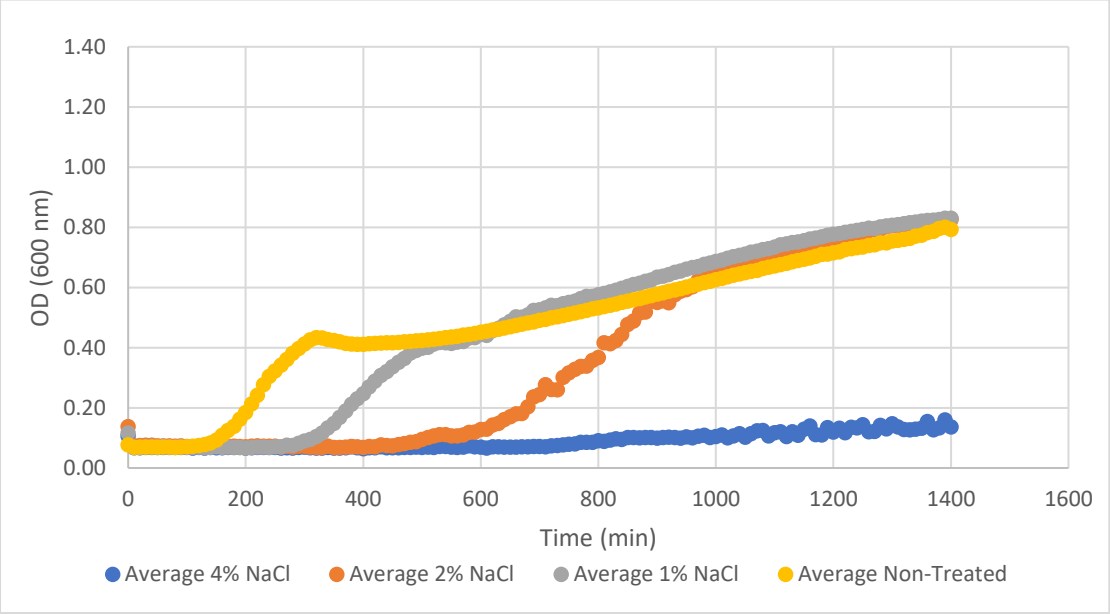
The effect of NaCl on the growth of *B. cereus* NVH spores is described in two replicated experiments as shown in Table 16. In both experiments, the highest TTD was found at salt concentration of 4% with 1740 min and 830 min respectively. Comparatively, the TTD at 4% NaCl had the longest followed by TTD at 2% NaCl and then 1% NaCl in both experiments. The non-treated sample which contained *B. cereus* NVH *spores* in LB without salt had the shortest TTD at 280 mins, illustrating the inhibitory effect of NaCl on the growth of bacterial spores in foods.

The inhibitory and antimicrobial properties of NaCl in food preservation systems have been studied (Doyle & Glass, 2010; Silva & Lidon, 2016). Conventionally, NaCl is known to be an important food preservative that inhibits or limits the growth of foodborne pathogens and spoilage organisms by decreasing water activity (a_w) (Jovanovic et al., 2021). A reduction in moisture content disrupts vital microbial processes by initiating changes in cellular metabolism. Higher concentrations of NaCl induces a hyper osmotic pressure in cellular environments leading to plasmolysis which inhibits the growth and development of putrefactive or pathogenic bacteria (Singh & Shalini, 2016). (Kim et al., 2017) investigated the effect of different concentrations NaCl on *B. cereus* growth in salted shrimps.

Averagely, the overall *B. cereus* counts in shrimp treated with 2, 5, 10, and 15% NaCl decreased significantly with the highest salt concentration (15%) showing the highest log reduction. This illustrates the impact of increasing NaCl concentrations on *B. cereus* growth (Kim et al., 2017). Existing data shows that at 7% NaCl about 11-89% of *B. cereus* strains can

grow although there have been some inconsistencies in these reports and hence the growth of *B. cereus* in NaCl is strain dependent (Authority, 2005; Raevuori, 1975). Therefore, growth at final sample concentrations of 4%, 2% and 1% was to be expected (Rajkowski & Bennett, 2003). According to (Raevuori, 1975), the detection time can be determined by the increasing concentrations of NaCl coupled with other factors such as the initial inoculum concentration, temperature, strain and media composition.

(A)



(B)

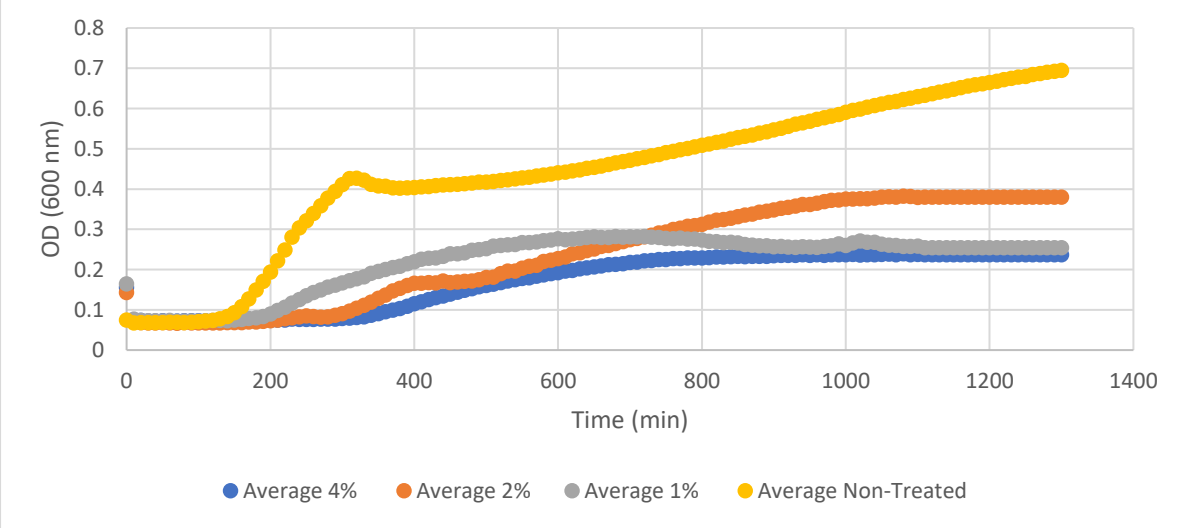


Figure 24: Effect of different NaCl concentrations on spores of B. cereus NVH *from a repeated experiment (A) and (B). The NaCl concentrations of 4%, 2% and 1% was compared to the non-*

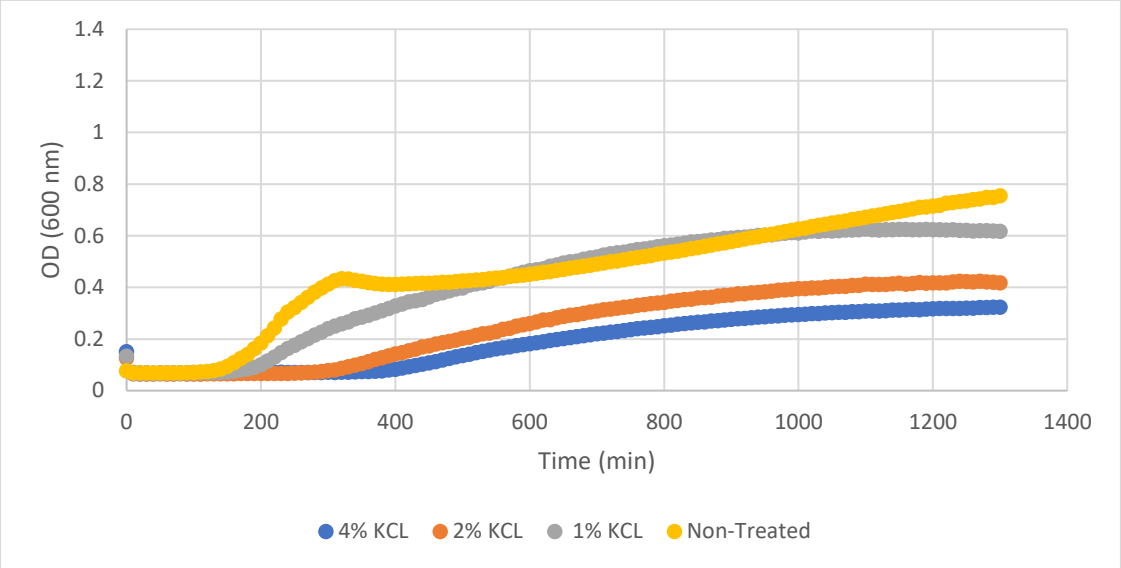
treated sample. TTD was longer in 4% NaCl than the non-treated sample. The data is mean values of 3 parallels collected from a repeated Bioscreen experiment.

4.2.2 Effect of KCl on *B. cereus* NVH spore growth

The Table 16 illustrates the effect of KCl on the growth of *B. cereus* NVH spores in two replicated experiments. The inactivation using KCl followed a trend similar to the use of NaCl where TTD was longer at 4% KCl followed by 2% and 1% KCl in both experiments compared to the non-treated sample. Even though the inhibitory effect in NaCl seem to have a longer TTD thus exerting a stronger inhibitory effect than KCl, research has shown the excessive use of NaCl has been associated with the increased risk of several cardiovascular diseases hence making KCl an ideal alternative (de Marco et al., 2022; Domínguez et al., 2017). Studies by Roeßler et al. (2003) pointed out that the inhibitory effect is mainly caused by the toxicity Cl^- ions in bacteria. At higher salt concentrations, Cl^- ions becomes essential for the growth of some *Bacillus* species. Some of these species exhibit strict chloride dependence which makes the presence Cl^- ions vital for the growth of these pathogens (Taormina, 2010).

KCl has been used as a substitute for NaCl in many food preservation systems. Research by Boziaris et al. (2007) have documented the effect equal-molar concentrations of NaCl or KCl on the growth kinetics of *Listeria monocytogenes*. According to this study, both NaCl and KCl exerted similar inhibitory effects against *Listeria monocytogenes* in terms of lag phase duration, growth or death rate. The study emphasized that NaCl can be replaced by KCl without the risk of microbiological hazard, with respect to *L. monocytogenes*, of the product. Reports by Bidlas and Lambert (2008) and Beuchat (1974) also stated that at equivalent a_w, NaCl and KCl had similar effects against *Vibrio parahaemolyticus*. On the contrary, some experiments have demonstrated the effect of solute identity on the amount of growth for a given a_w. According to Zarei et al. (2012) for the growth of *Clostridium perfringens*, KCl demonstrates a greater effect than NaCl. Based on these findings, the effect of KCl and NaCl is dependent on the type and strain of organism as well as the salt concentration (Bidlas & Lambert, 2008).

(A)



(B)

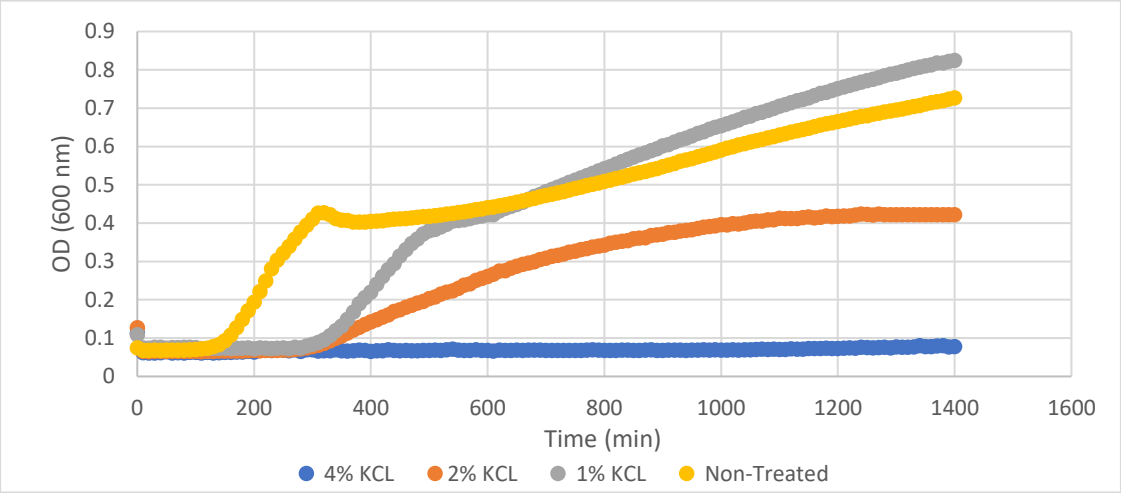


Figure 25: Effect of different KCl concentrations on spores of B. cereus NVH *from a repeated experiment (A) and (B). The KCl concentrations of 4%, 2% and 1% was compared to the non-treated sample. The data is mean values of 3 parallels collected from a repeated Bioscreen experiment.*

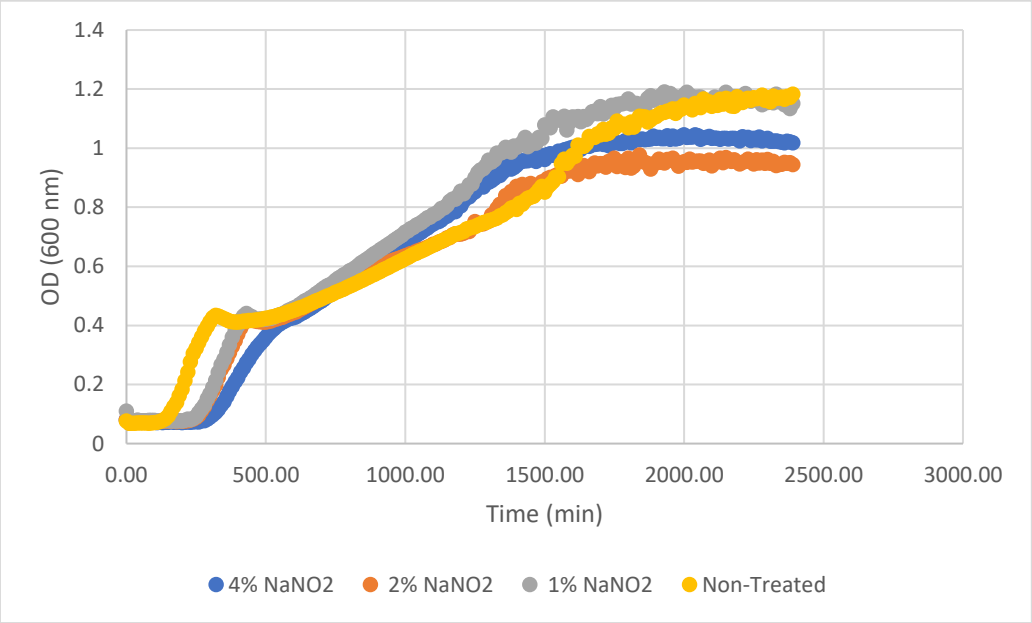
4.2.3 Effect of $NaNO_2$ on the growth of *B. cereus* NVH

The effect $NaNO_2$ on the growth of *B. cereus 144* spores is illustrated in Figure 26. According to the results, increasing concentrations of $NaNO_2$ (500, 125 and 62.5 ppm) had no effect on the growth and TTD of *B. cereus* NVH spores.

Sodium nitrite have been used in the food industry for many years for curing and preserving meats and fish, and in the manufacture of certain cheeses (Binkerd & Kolari, 1975; Bryan &

Ivy, 2015; Pegg & Shahidi, 2008; Tompkin, 2005). It is also responsible for the color changes in cured meats for example the pink color of cured hams (Bhusal et al., 2021). However, the application of nitrite is done in lower concentrations in compliance with food safety regulations as higher concentrations could be toxic for human (Lebelo et al., 2021; Sindelar & Milkowski, 2012). According to the European Union under Commission Regulation (EU) No 1129/2011, The amount of nitrite permitted for use in processed meat is currently 150 mg kg^{-1}, with the exception of sterilized meat products for which the limit is 100 mg kg^{-1} (Karwowska & Kononiuk, 2020). Extensive research has been carried out on the use of nitrite as a food preservative in meat products to prevent the growth and germination of heat-resistant spores of *C. botulinum*, and subsequent toxin formation (Bhusal et al., 2021). In contrast, the use of nitrite has proven to be least effective particularly against *S. aureus* in a study carried out by Thomas et al. (1993). In a study by Sofos et al. (1979), nitrite alone slowed down the rate of toxin formation in *C. botulinum*. However, a combination of nitrite and sorbate extended the time further. Also, sorbic acid (0.2%) alone or in combination with nitrite (20, 40, and 156 micrograms/g) significantly (P less than 0.05) inhibited spore germinations (Sofos et al., 1979).

(A)



(B)

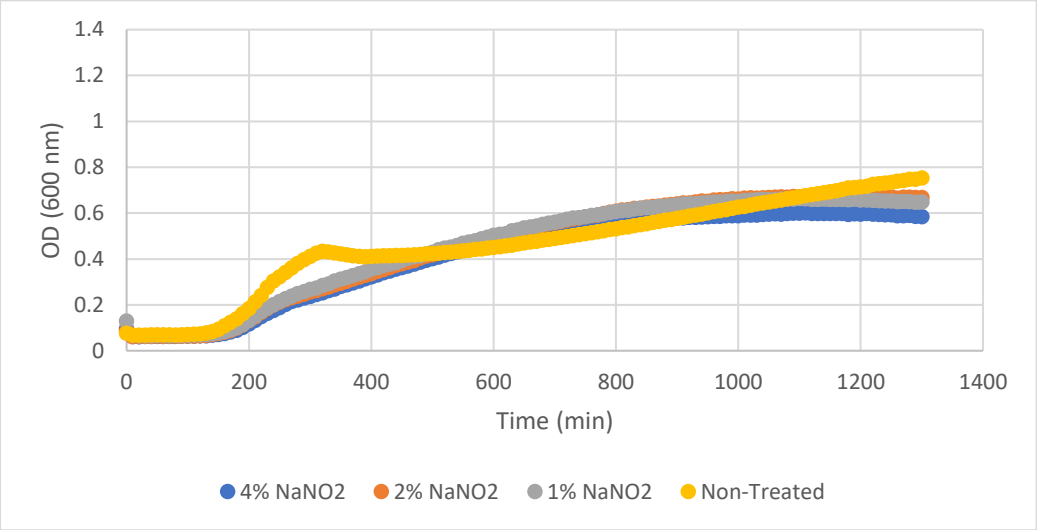


Figure 26: Effect of $NaNO_2$ on the growth of B. cereus NVH *spores from two repeated experiments (A) and (B). No significant difference in growth was observed in the varying concentrations of $NaNO_2$. The data is mean values of 3 parallels collected from a repeated experiment.*

4.2.4 Effect of pH on growth of *B. cereus* NVH spores

Effect of pH on growth of *B. cereus* NVH was studied. According to the results from Table 16, pH 7 exerted the least inhibitory effect on *B. cereus* NVH with the least TTD in both experiments A and B. In experiment A, growth at pH 4 was observed while in experiment B, no growth at pH 4 was seen. However, literature reports indicate that *B. cereus* can grow in a pH range between 4.5 to 9.5 (Rodrigo et al., 2021). Therefore, growth at pH 4.0 was least expected in our experiment. To validate the discrepancies in results between the two experiments, another confirmatory test to determine the growth of *B. cereus* NVH at pH 4 was carried out using Bioscreen and observed under the microscope. No growth of *B. cereus* NVH vegetative cells at pH 4 was observed in this test, confirming the results of experiment B. The growth at pH 4 in experiment A could be attributed to the presence of elongated cells or rods at lower pH (pH 4) in accordance with a report by (Everis & Betts, 2001). This research suggested that subjecting spore-formers to the environmental stress of low pH could result in an increased cell length. In another study, increase the length of the *B. cereus* strain was revealed after exposing to pH 5 (Jobin et al., 2002). Reports by Browne and Dowds (2002) and Jobin et al. (2002) have also demonstrated the ability of some *B. cereus* strains to survive under harsh environmental conditions such as low pH (1 to 5.2) by acid tolerance mechanisms (Vidic et al., 2020). However, reports by EFSA (2005) indicate that *B. cereus* is not particularly an

acid tolerant bacterium (Authority, 2005). Experiment by Valero et al. (2003) demonstrated that the pH limit for growth in carrot substrate acidified with citric acid was between 4.5 and 4.75. Tewari and Abdullah (2015) reported that foods with pH less than 4.3 can be considered safe from growth of the foodborne illness *Bacillus* species.

In experiment B, growth was observed at pH 5 through to pH 7 as expected according to literature. Research shows that, pH ranges between 4.5 to 9.5 promotes *B. cereus* growth. However, with a synergistic combination of appropriate hurdles, growth of *B. cereus* within this ranges can be inhibited. For example, in an experiment by Valero et al. (2003), the combination of mild acidification (pH 5.0) and refrigeration (≤8 °C) inhibited *B. cereus* growth in vegetable substrates for at least 60 days. Figure 27 further illustrates the growth of *B. cereus* at respective pH values from 4 to 7 as described in Table 16.

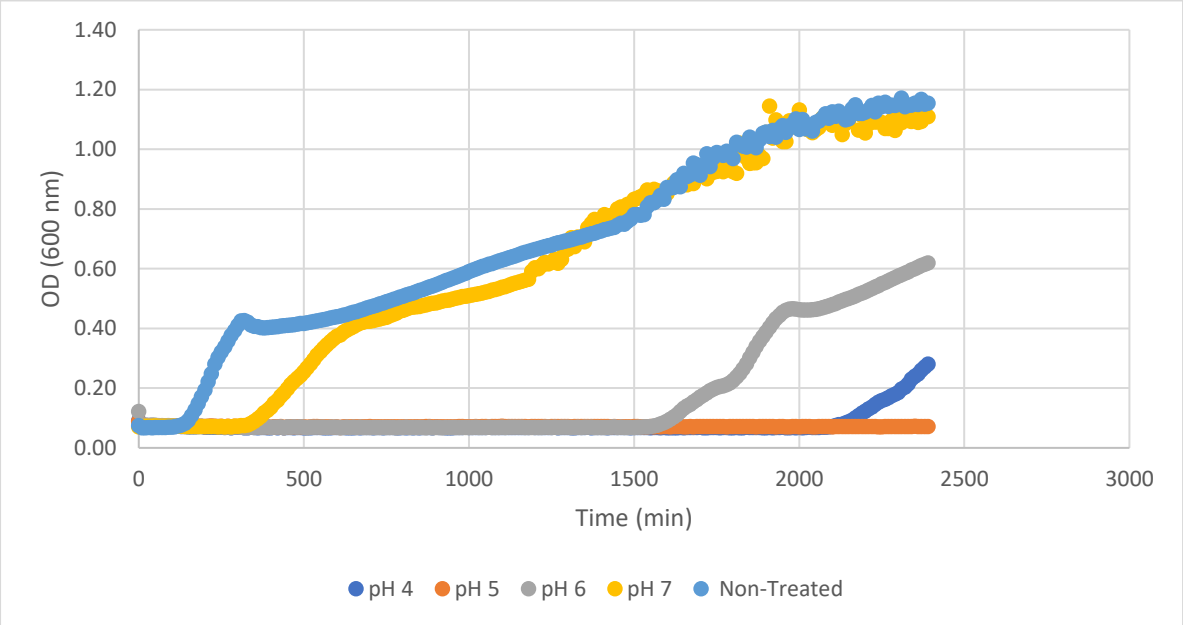


Figure 27: Effect of pH 4, 5, 6, and 7 on B. cereus NVH *growth.*

4.2.5 Confirmatory Test of *B. cereus* NVH growth at pH 4

The growth of *B. cereus* NVH vegetative cells at pH 4 was investigated. No growth at pH 4 was observed in this experiment. This was similar to research works by Rodrigo et al. (2021) who proposed that *B. cereus* can grow within a broad pH range between 4.5 to 9.5. Their study stated that vegetative cells of *B. cereus* die immediately below pH 4.3 and therefore no growth was expected at pH 4.

4.3. Inactivation of spores by heat treatment of spores in LB medium

Thermal inactivation of *B. cereus* spores was carried out in three separate experiments (A, B and C). The first experiment (A) involved thermal inactivation at 70°C and 80 °C for 5 and 10 mins and 95°C for 5 mins respectively. In experiment (B), thermal inactivation was carried out at 80°C, 90°C and 95°C for 5 mins and 10 mins respectively. Experiment C was conducted at 95°C and 97°C for 5 and 10 mins respectively. Figure 28 shows the log reductions of the spores heated at selected temperatures and times in experiment A.

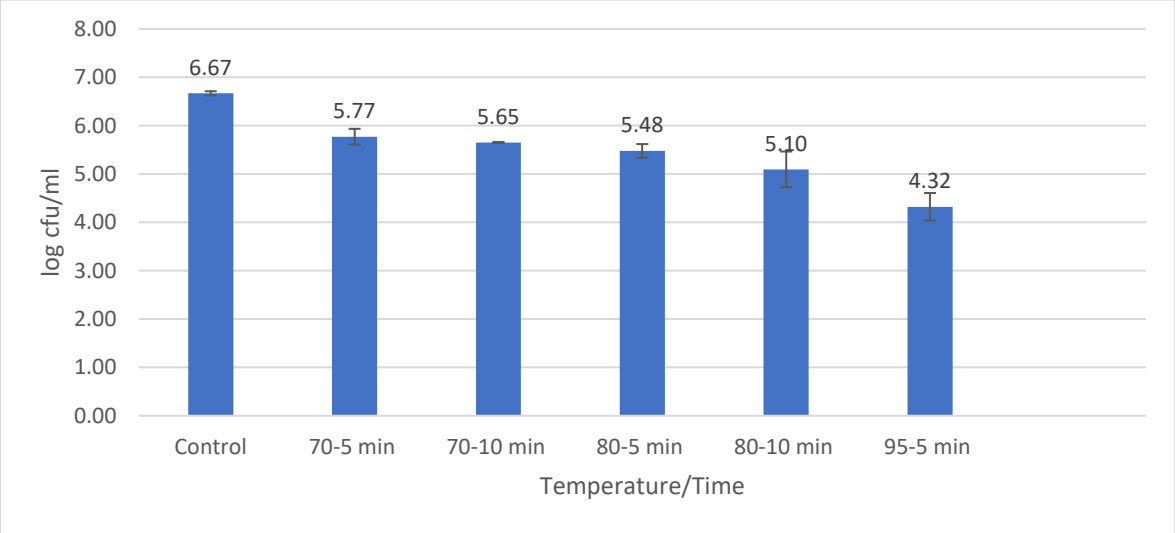


Figure 28: Spore inactivation and log reductions of B. cereus NVH *spores at 70°C, 80 °C and 5 and 10 mins and 95°C for 5 mins in LB medium.*

The highest spore inactivation was achieved at 95°C for 5 mins while the lowest spore inactivation was observed at 70°C for 5 mins.

Compared to the control, a log reduction of 2.35 log was obtained at 95°C for 5 mins while at 70°C for 5 and 10 mins, a log reduction of 0.9 log and 1.02 were achieved. At 80°C, 1.19 and 1.57 log reductions were achieved at 5 mins and 10 mins respectively. It could be observed that an increase in temperature with a corresponding increase in time resulted in a high spore inactivation with increased log reductions.

A similar trend was also observed in experiment B, where thermal inactivation of *B. cereus* spores was tested at 80°C, 90°C and 95°C for 5 and 10 mins. Here, the highest spore inactivation was achieved at 95°C for 10 mins with 4.51 log cfu/ml while the lowest spore inactivation of 6.23 log was observed at 80°C for 5 mins. At 90°C for 5 and 10 mins, a log reduction of 0.7 log and 1.07 were achieved. At 80°C, 0.5 log and 1.41 log reductions were achieved at 5 mins and 10 mins respectively. Contrary to the first experiment, a higher spore

inactivation was observed at 80℃ for 10 mins than for 90℃ for 10 mins which was not as expected.

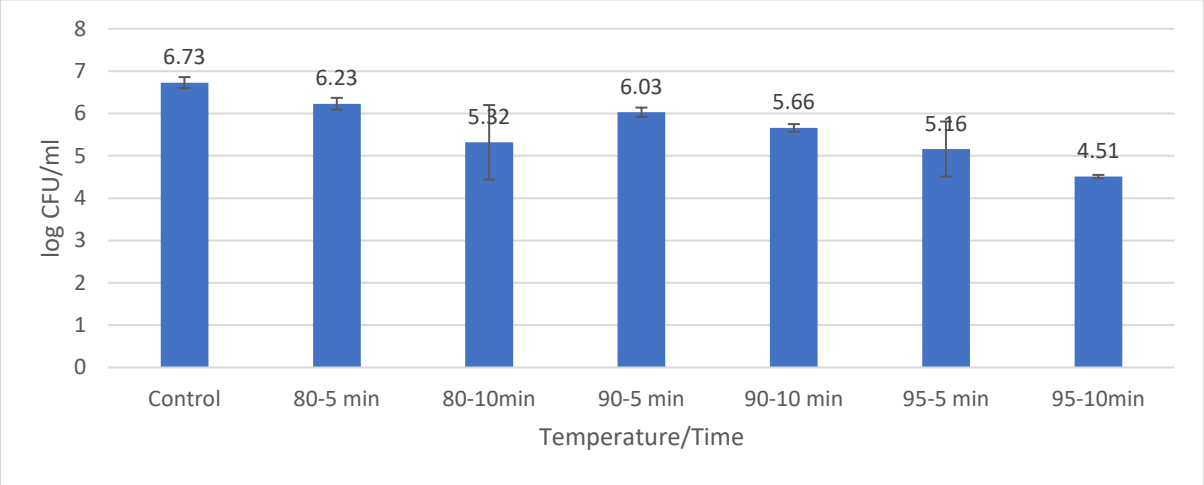


Figure 29: Log reductions of B. cereus NVH *spores at 80℃, 90 °C and 95℃ for 5 and 10 mins in LB medium in experiment B.*

Due to the fact that spore inactivation of *B. cereus* NVH was high at 95℃ for 5 and 10 mins, it was proposed that the *B. cereus* NVH strain was heat resistant. A third experiment with a higher temperature than 95℃ (97℃) was carried out to ascertain the heat resistance of the *B. cereus* NVH strain. Figure 30 further describes the log reductions of *B. cereus* NVH spores at 95℃ and 97℃ for 5 and 10 mins in LB medium.

In Figure 30, a higher spore inactivation was achieved at 97℃ compared to 95℃. Comparing this to the control experiment, a log reduction of 0.94 log cfu/ml and 3.02 log cfu/ml were obtained at 95℃ for 5 and 10 mins respectively. At 97℃ for 5 and 10 mins, there was an increase in log reduction of 3.74 log cfu/ml and 4.10 log cfu/ml respectively. This reemphasized the effect of high temperature/time profiles on spore inactivation.

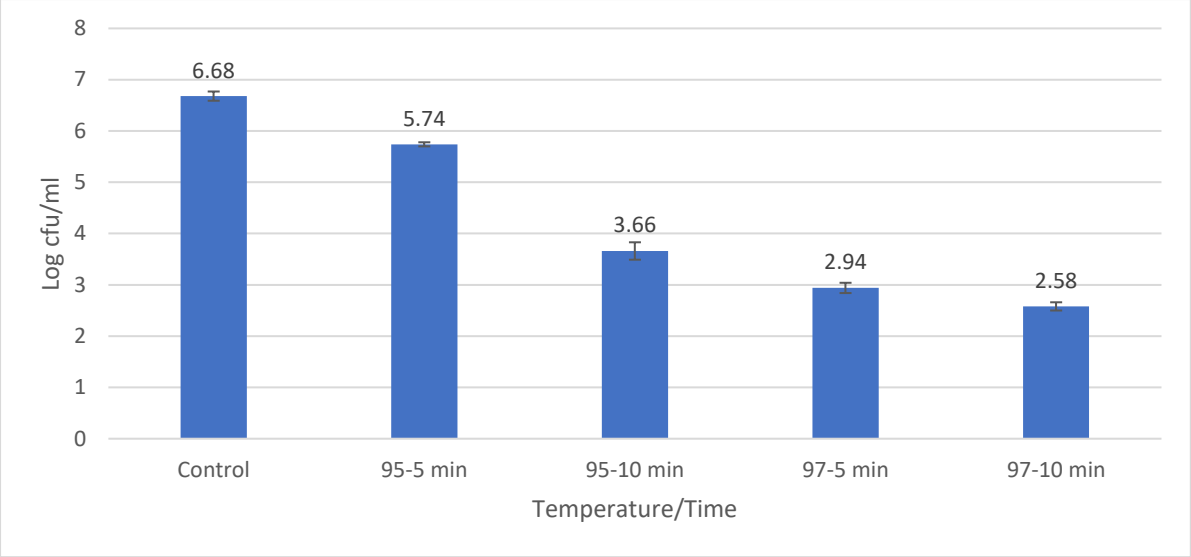


Figure 30: Log reductions of B. cereus NVH *spores at 95 °C and 97°C for 5 and 10 mins in LB medium.*

Thermal inactivation is the most common method used to control microbial contamination in many food processing industries. However, the resistance of bacterial spores to heat has become a huge food safety concern as the application of sublethal heat treatment may activate dormant spores and initiate the conversion of spores to vegetative cells (Løvdal et al., 2011). Studies by Wells-Bennik et al. (2016) indicated that the application of temperatures between 65 and 95°C were inadequate to kill all bacterial spores but enough to eliminate competing vegetative cells (Wells-Bennik et al., 2016). In view of this, research by Løvdal et al. (2011) proposed a double heat treatment model in controlling *B. cereus* spores based on the fact that germinated spore have a lower heat resistance than dormant spores. In this study, *B. cereus* NVH spores supplemented in food model matrices, heat activated at 70°C and then heat inactivated at 80 or 90°C resulted in a significant increase in log reduction.

Research has revealed specified D-values for many bacterial species and strains. This is the time required at any specific temperature to inactivate 90% of the bacterial population (Byrne et al., 2006). However, the heat resistance of bacteria is dependent on many factors including the strain, sporulation temperature, matrix composition (broth or food matrix), spore structure and mechanism of germination (Setlow, 2010). High temperature time profiles have an influence on spore inactivation and log reductions. In a study by Evelyn et al., (2022), the thermal inactivation of B. subtilis and B. licheniformis spores in pineapple juice at different temperatures (85–100°C) were investigated. A linear decrease in D-value was observed with increasing temperature of treatment (Evelyn et al., 2022). An experiment by Daelman et al.

(2013) compared the effect of three heat treatments at different temperatures (85, 87 and 90 °C), on the germination and subsequent growth of *B. cereus*. The results showed that lower heating temperatures (85 and 87 °C) had less effect on the TTG (time to growth), minimal a_w and pH than a higher temperature (90 °C), illustrating the effect of high temperatures on bacterial growth. A study by Desai and Varadaraj (2010) also demonstrated the effect of different temperatures on *B. cereus* vegetative cell and spore growth and how these temperatures affected D-values of the bacteria. Results on vegetative cells showed that D-values at 60℃, 58℃ and 56 ℃ in cultures and heating media were 3.8, 4.9 and 7.4 mins respectively ($p \leq 0.05$), indicating that high temperatures contributed to a decrease in D-values with a corresponding decrease in bacterial growth. Works by Byrne et al. (2006) have also illustrated how *B. cereus* and C. perfringens spores and vegetative cells are inactivated at specified time/temperature profiles. In this study, pork luncheon roll was inoculated with *Bacillus cereus* vegetative cells and subjected to thermal treatments at different temperatures. The *D*-values ranged from 1 min (60 °C) to 33.2 min (50 °C) for *B. cereus.* However, an increase in temperature to 105℃ at 36 s resulted in a 6 log reduction of *B. cereus* spore, also demonstrating the effect of high temperatures on bacterial growth reduction.

Research works by Silva (2020) also indicated that the inactivation of *B. cereus* spores in beef slurry, rice porridge and cheese slurry gave a 0.8 log reduction at 70℃ alone compared to a combination of thermal treatment and other hurdles which resulted in higher log reductions ($p < 0.05$). From the results of this study, higher temperature/time profiles especially at 90℃, 95℃ and 97℃ resulted in higher log reductions with increased spore inactivation in accordance with literature. However, less difference in log reductions were observed at 70℃ and 80℃ as described in Figure 28.

4.4 Inactivation of spores in food matrix by heat treatment alone and pressure-thermal treatments (PATS).

The inactivation of *B. cereus* NVH spores by thermal treatment alone and thermal treatment coupled with high-pressure processing was investigated in minced meat.

4.4.1 Inactivation of spores by heat treatment

The effect of thermal treatment on *B. cereus* spore reduction was investigated. Minced meat was used as a food matrix. The meat was inoculated with spores and heated at 97℃ for 10 minutes to assess the effect of heat on spore reduction. The results were compared to a control experiment with an initial spore concentration of 10^7 log cfu/ml. A replica experiment was

conducted and the average log reductions of the two experiments (six parallels) were used to produce graphs as shown in Figure 31.

The inactivation of *B. cereus* NVH spores in minced meat at 97°C for 10 minutes gave a log reduction of 3.35 log cfu/ml compared to the control experiment. The result of this experiment reveals the effect of matrix composition on spore thermal resistance. When spores were heated at 97°C for 10 minutes in LB medium, a log reduction of 4.1 log cfu/ml was obtained. Under the same thermal conditions in minced meat, the log reduction was relatively lower (3.35 log cfu/ml), illustrating the shielding effect of food matrices on spore thermal resistance. According to Georget et al. (2015) and Sevenich and Mathys (2018) food matrices provide complex environments which may offer shelter to microorganisms, even under harsh treatment conditions. Particularly, low water activity (a_w) matrices have demonstrated challenges to achieve microbial decontamination by any kind of decontamination strategies, including HPP (Gurtler et al., 2014; Tapia et al., 2020). Other literature studies have also reported that salt and sucrose media provided an enhanced protection for bacterial spores (Considine et al., 2008; Georget et al., 2015; Rendueles et al., 2011).

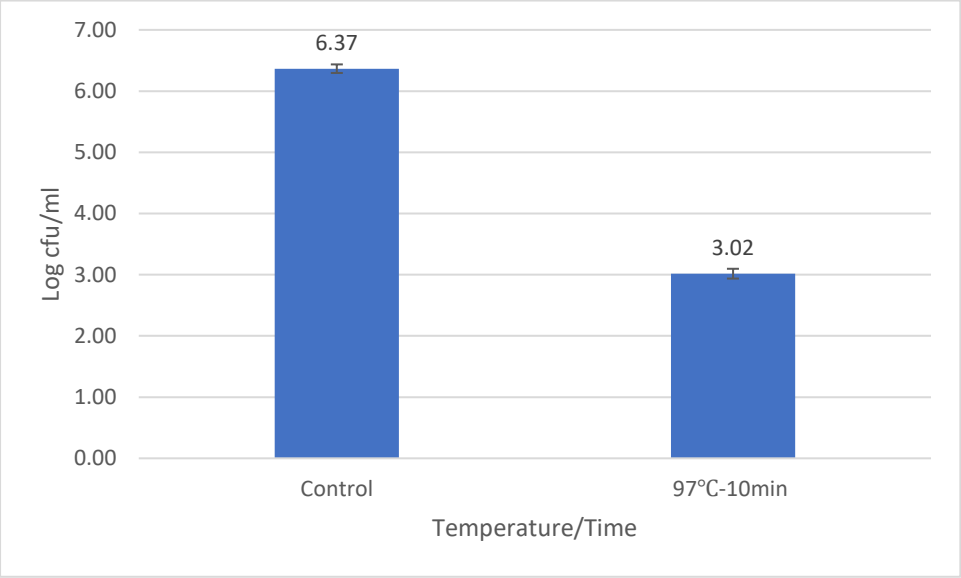


Figure 31: Inactivation of B. cereus spores in minced meat after heat treatment at 97 °C for 10 minutes. The data is mean values of six parallels and the standard deviations are shown for each treatment.

4.4.2 Inactivation of spores by PATS

The effect of spore inactivation by HPP at three different processing temperatures on the *B. cereus* spores were investigated. Sample packages containing 6 g of inoculated minced meat

were subjected to a pressure of 600 MPa at 20, 40 and 55 °C. All three pressure-temperature combinations were studied at a holding time of 2 mins. Each treatment was performed in triplicate and the experiment was repeated twice. The average log reductions of the six parallels in both experiments were used to obtain a graph where spore log reductions (log N_0/N) were plotted against treatment condition (Temperature/Time) as illustrated in Figure 32. Spore logarithmic reductions were also compared with the results of thermal treatment at 97℃ for 10 mins and a control experiment to reveal differences in both thermal and non-thermal processing operations.

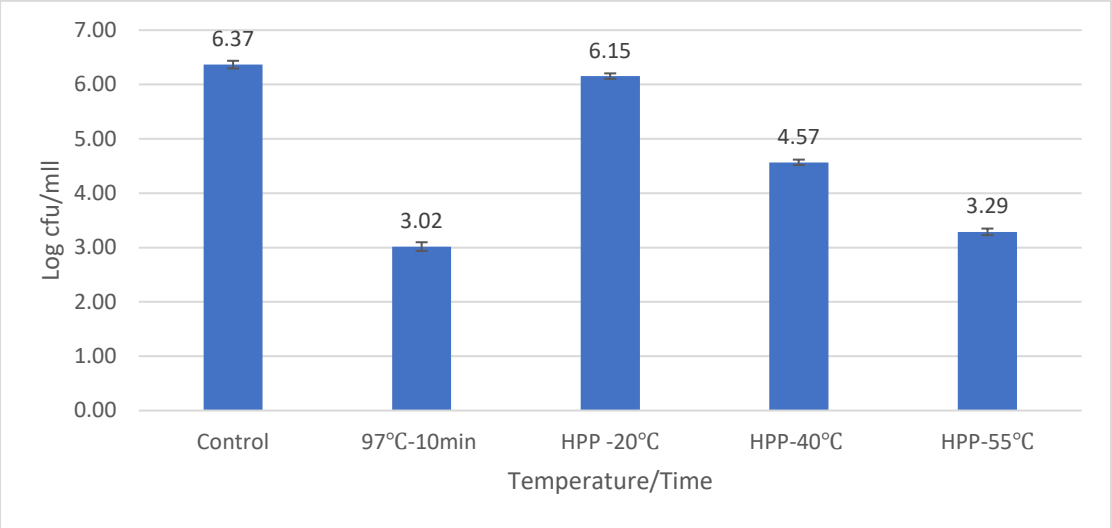


Figure 32: Inactivation of B. cereus spores in minced meat after HPP processing at 600 MPa at different temperatures.

The highest spore inactivation by pressure-thermal treatment was recorded at HPP-55℃ for 2 min with 3.08 log cfu/ml, followed by HPP-40℃ with 1.80 log cfu/ml. The lowest spore inactivation was recorded at HPP-20℃ for 2 min with 0.22 log cfu/ml. The results showed that HPP at 20℃ had the lowest spore inactivation (≥3 log reduction). Also, not much difference in spore inactivation were observed at HPP 55 °C and thermal treatment alone at 97°C. Spore inactivation of 3.08 and 3.35 log cfu/ml reductions respectively were observed for the HPP–thermal process (600 MPa-55°C) and thermal process (97°C).

A similar experiment was conducted by Evelyn and Silva (2016) where an increase of HPP temperature from 38℃ to 70℃ doubled log reductions in *B. cereus* in beef slurry. In their study, the potency of 600 MPa HPP in combination with 70 °C for the inactivation of *B. cereus* ICMP 12442 spores in beef slurry was investigated and compared with 70 °C thermal processing alone. The HPP-70 °C process enhanced the *B. cereus* spore thermal inactivation in beef slurry, resulting in 4.9 log cfu/ml reductions after 20 min vs. 0.5 log cfu/ml for thermal processing alone. In another experiment by Evelyn and F. V. M. Silva (2015), the same HPP-

thermal conditions were applied in milk. HPP-thermal temperatures from 38℃ to 70℃ tripled *B. cereus* log reductions in milk. Experiments by Balasubramanian and Balasubramaniam (2009) also demonstrated the effect of buffers and food substrate on spore inactivation and concluded that the buffers had a significant influence on the spore inactivation compared to the food substrate. The highest inactivation (≥7 $\log_{10}$ units) was obtained when the spores suspended in buffer were processed at 827MPa–75C for 5min. Research by Van Opstal et al. (2004) also showed that when HPP was coupled with mild temperatures, spore inactivation of > 5 log were achieved at 500 MPa pressure at 60 °C. Oh and Moon (2003) also highlighted that high processing temperatures during HPP enhanced the effect of sporulation medium pH on *B. cereus* spore inactivation.

With reference to thermal treatments, *B. cereus* spores also exhibit resistance to HPP treatments. Differences in spore responses to HPP can be caused by intrinsic and extrinsic factors such as bacterial strain, sore structure, sporulation conditions, medium nutrients, ionic strength, pH and pressure-transmitting fluid (Olivier et al., 2012). The application of pressure initiates germination which makes germinated spores more sensitive to agents such as heat, pH and pressure than they were in dormant state (Paidhungat et al., 2002). However, the optimum pressure and temperature for germination depends on species, pressure, suspending medium, and storage time after heat activation. In order to overcome spore resistance by HPP, several proposed combination treatments like double heat treatments, PATS, etc. have been used to achieve appreciable spore inactivation (Løvdal et al., 2011; Lv et al., 2021; Raso & Barbosa-Cánovas, 2003).

4.5 The combined effect of preservatives on *B. cereus* NVH spore inactivation

The effect of combining different preservatives at different concentrations on the growth of *B. cereus* NVH spores was investigated using the Bioscreen C. In this experiment, NaCl at different concentrations were used with different pH values and the effect of this combination on the growth of *B. cereus* NVH spores was determined. Thereafter, NaCl and $NaNO_2$ at different concentrations were used at different pH values and the combined effect of these preservatives on the growth of the bacterial spores were also examined. The Table 17 and Table 18 describes the Time to detection (TTD) of each combined preservative at different concentrations in two replica experiments.

4.5.1 Effect of NaCl and pH on *B. cereus* NVH growth

According to Table 17 and Table 18, no TTD values were recorded at high salt concentrations (4% and 2% NaCl) and pH 4. However, in experiment B, a TTD of 293 mins was recorded at

2% NaCl at pH 5 indicating the possibility of bacterial growth. Similarly, both experiments revealed growth at lower NaCl concentrations (2% NaCl and 1% NaCl) at pH 6. Higher TTD values were also obtained at 2% NaCl compared to 1% NaCl at equal pH indicating the importance of NaCl concentration in spore growth inhibition. For example, in both experiments, 2% NaCl at pH 6 gave a TTD of 3210 mins and 2840 mins respectively while 1% NaCl at pH 5 gave a TTD of 300 mins and 700 mins respectively. The experiment also showed that a combination of NaCl at lower concentrations of 2% or 1% with pH 4 or pH 5 was not enough to inactivate the growth of *B. cereus* NVH spores. Probably, the introduction of more hurdles could potentiate the inactivation of *B. cereus* NVH spores. The results of this experiment evidently illustrate that a combination of NaCl at high concentrations with lower pH exerts a significant inhibitory effect on the growth of *B. cereus* NVH spores.

A study by Martínez et al. (2007) demonstrated the combined effects of temperature, pH and NaCl concentration on the growth kinetics of *B. cereus* ATCC 7004, after mild heat treatment in nutrient broth and in a meat extract. In this study, the influence of temperature (10 to 50 °C), initial pH (4.0 to 6.0) and sodium chloride concentration (0.5 to 3.0%) on the growth in nutrient broth and in meat extract of *Bacillus cereus* after mild-heat treatment (90 °C—10 min) was investigated. Results of the experiments indicated that a decrease in pH values with an increase in of sodium chloride concentrations decreased growth rate and increased the lag phase of *B. cereus*. Relative to our experiment, a similar trend was observed in our study where at 4% NaCl at pH 4, no growth was observed for *B. cereus* NVH spores. The study also revealed that pH 4.5 could not prevent the growth of spores in a meat substrate with 0.5% NaCl which was also true for our experiment where pH 5 and pH 6 (above pH 4) exerted less inhibitory effect at the lowest NaCl concentration of 1%. According to their study, the combined effect of acidification ($pH \leq 4.5$) and NaCl concentration of $\geq 1\%$ was sufficient to prevent the growth of heated *B. cereus* ATCC 7004 spores in nutrient broth and meat extract during storage for at least 50 days. In our experiment, the efficiency of lower pH values combined with high salt concentrations was similarly emphasized where at 4% NaCl at pH 4, no growth was observed in both experiments. GonzÁLez et al. (1997) showed that increasing the salt levels from 0.5 to 4% led to a progressive decrease in spore recovery. D-values decreased gradually as salt content increased.

4.5.2 Effect of NaCl and $NaNO_2$ on *B. cereus NVH* growth

Results from Table 17 and Table 18 showed that the least inhibitory effect on the growth *of B. cereus* NVH spores was achieved at 1% and 2% NaCl combined with 1000 $NaNO_2$, pH 6. On

the other hand, the results indicated a high spore growth inhibition at 4% and 2% NaCl combined with1000 $NaNO_2$, pH 4 and pH 5 respectively. No growth or increase in TTD at 0.2 was recorded at these combinations. Based on the previous results on NaCl and pH, it was stated that high salt concentrations (4% and 2% at pH 4 and 5) had the most inhibitory effect on the growth of *B. cereus* NVH. Therefore, the addition of a 1000ppm $NaNO_2$ to NaCl and pH played no significant role in enhancing the inhibitory effect on the growth of the spores.

Sodium nitrite has been widely used as a food preservative in ready-to-eat meats. Nitrite aids in flavor development, reacts with myoglobin to produce nitrosylhaemochrome, which gives processed meat its distinctive pink color, and inhibits the growth of spoilage and pathogenic bacteria such as *Clostridium botulinum* (Lim et al., 2016). Other pathogens such as *Bacillus cereus, Staphylococcus aureus,* and *Clostridium perfringens* may be inhibited by nitrite in combination with other salts and curing factors (Pradhan et al., 2009). However, it does not prevent pathogen outgrowth under conditions of prolonged temperature fluctuations in a range that promotes bacterial growth and may allow toxin production or spoilage (Parthasarathy et al., 2012). Research by Lim et al. (2016) investigated the minimum inhibitory concentrations for sodium nitrite, sodium nitrate and sodium metabisulphite against *C. difficile*. The results indicated that the modal MIC values for sodium nitrite, sodium nitrate and sodium metabisulphite were 250 μg/ml, >4000 μg/ml and 1000 μg/ml, respectively. No bactericidal activity was observed for all three food preservatives.

Table 17: Time to detection of combined preservatives at different concentrations used in experiment A.

Preservative	Concentration (%)	TTD (min)	max OD	Time to max
NaCl, pH 4	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	2490	0.49	2946
NaCl, pH 5	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	1550	1.02	3400
NaCl, pH 6	4%	n.g	n.g	n.g
	2%	3210	0.9	3550

	1%	390	1.04	2310
NaCl, 1000 $NaNO_2$, pH 4	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	2590	0.67	3530
NaCl, 1000 $NaNO_2$, pH 5	4%	n.g	n.g	n.g
	2%			
	1%	1880	0.93	3450
NaCl, 1000 NaNO2, pH 6	4%	n.g	n.g	n.g
	2%	1630	0.65	3190
	1%	450	0.97	2320

***Where no growth was observed, 'n.g' was used to represent this.*

Table 18: Time to detection of combined preservatives at different concentrations used in experiment B.

Preservative	Concentration (%)	TTD (min)	max OD	Time to max
NaCl, pH 4	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	2210	0.37	3100
NaCl, pH 5	4%	n.g	n.g	n.g
	2%	293	n.g	n.g
	1%	1540	1.01	3110
NaCl, pH 6	4%	n.g	n.g	n.g
	2%	2840	0.3	3810
	1%	700	1.06	3670

NaCl, 1000 $NaNO_2$, pH 4	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	2600	0.62	3820
NaCl, 1000 $NaNO_2$, pH 5	4%	n.g	n.g	n.g
	2%	n.g	n.g	n.g
	1%	2940	0.61	3870
NaCl, 1000 $NaNO_2$, pH 6	4%	n.g	n.g	n.g
	2%	3320	0.36	3690
	1%	720	0.59	3390

***Where no growth was observed, 'n.g' was used to represent this.*

4.6 Psychrotrophic growth of *B. cereus NVH* vegetative cells at 8°C and 15°C combined with preservatives using Eddy Jet plating and Bioscreen.

The vegetative cell growth of *B. cereus* NVH was tested at 8°C and 15°C with a combination of preservatives (NaCl adjusted at pH 4) using the Eddy Jet method. The average initial bacterial cell number (bacterial starting number) was enumerated to $1.3x10^4$ cfu/ml by the Eddy Jet plating method. Bacteria suspended in LB medium (LB+Bac) with no preservatives was used as control.

Results showed that there was an increase in bacterial log numbers in samples with no preservatives at 8°C and 15°C as time progressed from day 0 to day 21. However, a higher increase in bacterial log numbers was recorded at 15°C compared to 8°C. On the contrary, there was a lower increase in bacterial log numbers in samples with 1% NaCl at 8°C at day 14 and 21 compared to samples with no preservatives at the same temperature and time.

Plates with 1% NaCl, pH 4 and 1% NaCl, pH 5 showed no growth per sample volume at day 21 at 8°C. On the other hand, there was an increase in bacterial log numbers in the same plates at 15°C, illustrating the effect of temperature and time even in the presence of preservatives. It

can be deduced that the efficacy of preservatives may decrease with time at high refrigeration temperatures. As time progressed, 1% NaCl was not enough to inhibit bacterial growth.

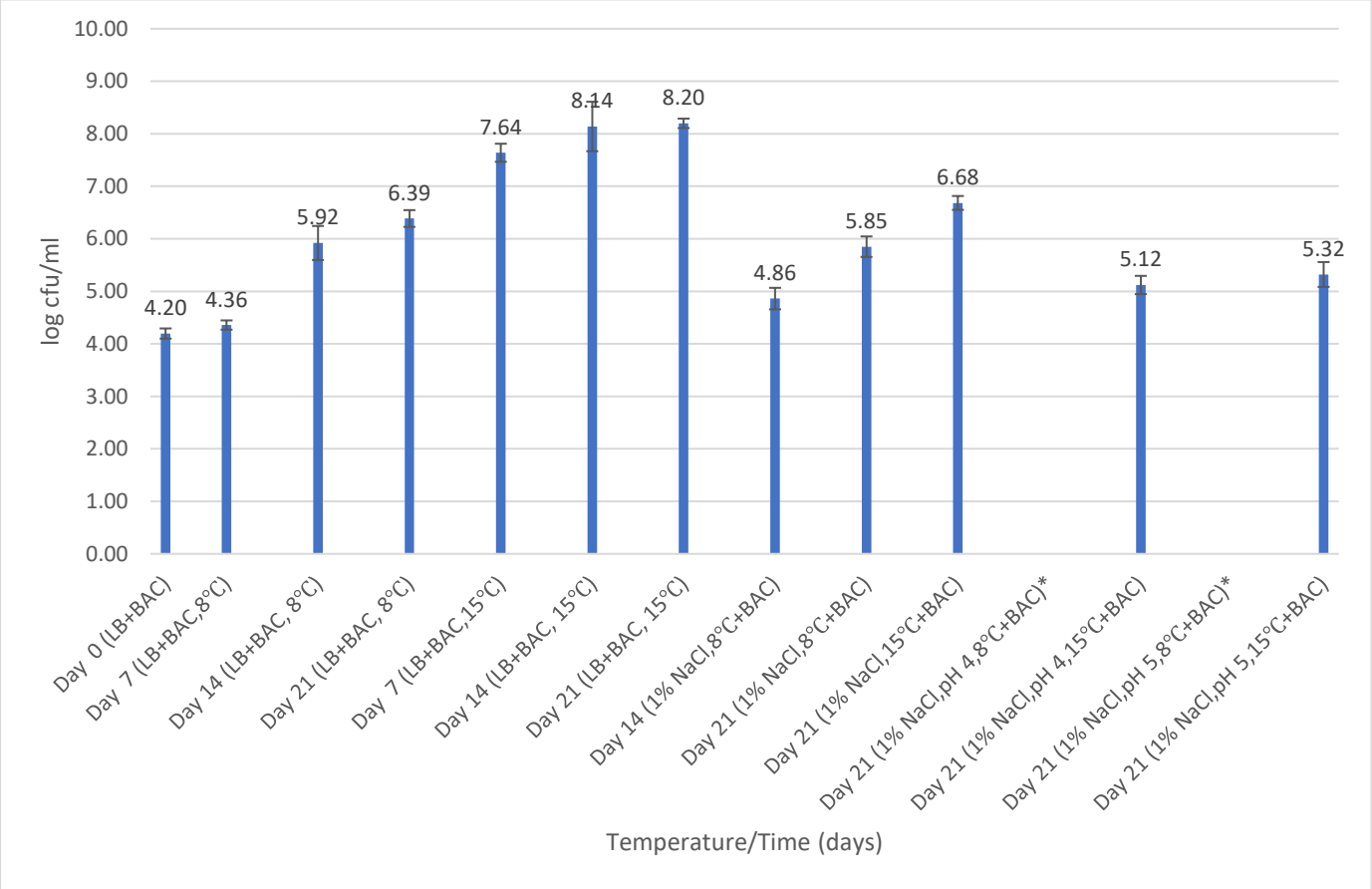


Figure 33: Effect of temperature and preservatives on bacterial log numbers with time.
**No growth on plates per sample volume.*

Results from Table 19 and Table 20 in Bioscreen (run at 20℃) also showed a similar trend where there was an increase in initial OD after incubation at 8℃ and 15℃ in wells with no preservatives as time increased. However, initial OD at 15℃ was higher compared to 8℃ in wells with no preservatives. On the other hand, there was a decrease in bacterial growth in wells with preservatives. Temperature and time played an important role in the extent of inhibition caused by the preservatives. For instance, 1% NaCl at pH 4 exhibited the most inhibitory effect on bacterial growth followed by 1% NaCl at pH 5 and 1% NaCl but the extent of inhibition was different at 8℃ and 15℃ as time progressed. The degree of bacterial growth inhibition was higher at 8℃ with preservatives than at 15℃ with preservatives as time increased.

Table 19: Effect of temperature/time and preservatives on initial OD after incubation at 8°C. Results are the averages and standard deviations of six parallels.

Preservatives (8°C)	Day 0	Day 7	Day 14	Day 21
Average LB	0.091	0.112	0.114	0.132
std	0.007	0.006	0.015	0.031
Average LB+Bac	0.349	0.139	0.163	0.171
std	0.00	0.021	0.026	0.013
Average 1% NaCl, no pH	0.081	0.090	0.105	0.111
std	0.009	0.009	0.013	0.037
Average 1% NaCl, pH 4	0.082	0.087	0.097	0.114
std	0.004	0.013	0.015	0.028
Average 1% NaCl, pH 5	0.083	0.084	0.110	0.114
std	0.009	0.010	0.043	0.031

Table 20: Effect of temperature/time and preservatives on initial OD after incubation at 15°C. Results are the averages and standard deviations of six parallels.

Preservatives (15°C)	Day 0	Day 7	Day 14	Day 21
Average LB	0.091	0.126	0.306	0.122
std	0.007	0.062	0.461	0.041
Average LB+Bac	0.349	0.549	0.754	0.703
std	0.008	0.090	0.064	0.327
Average 1% NaCl, no pH	0.081	0.400	0.414	0.363
std	0.009	0.078	0.129	0.073
Average 1% NaCl, pH 4	0.082	0.083	0.092	0.128
std	0.004	0.006	0.008	0.030

Average 1% NaCl, pH 5	0.083	0.083	0.092	0.103
std	0.009	0.013	0.014	0.030

Reports from the EFSA, (2005) documented psychrotropic growth of *B. cereus* below 10℃. Mesophilic strains were also reported to grow between 10℃ and 42℃ (Authority, 2005). Other studies have reported psychrotrophic growth temperatures at 4℃ or below (Luu-Thi et al., 2014). Studies show that the ability to grow at ≤8 °C is restricted to *B. cereus* phylogenetic groups II, V and VI and the capability of *B. cereus* strains from these three groups to grow at refrigeration temperatures is variable (European Food Safety Authority, 2016). Our study shows that *B. cereus* NVH was able to grow at 8℃ with time and hence could be described as psychrotrophic. *B. cereus* is known to be a significant hazard for minimally processed and refrigerated foods (Webb et al., 2019). Therefore, inappropriate storage practices, such as exposure to extreme temperatures or improper cooling provide opportunities for mesophilic strains of *B. cereus* to replicate (Français et al., 2021). Recommendations by EFSA revealed that to avoid the prevalence of *B. cereus* strains in foods, pH < 4.5, water activity <0.92 and storage temperatures <4 °C is recommended. Temperatures less than 10 °C significantly increased lag and generation times of *B. cereus*, especially under sub-optimal conditions.

4.7 The combined effect of preservatives and HPP on *B. cereus* spore growth in food

The combined effect of preservatives and HPP on spore inactivation by HPP were investigated. In this experiment, nisin at concentrations of 100 IU and 500 IU were combined with 2% NaCl at pH 4 and subjected to pressure-thermal treatments of HPP at 55℃ in minced meat samples. Results of each treatment was performed in triplicate and the experiment was repeated twice. The average log reductions of in both experiments were used to obtain a graph where spore log reductions were plotted against treatment conditions.

According to the results of experiment (A), the highest spore inactivation and log reduction were observed in meat samples treated with 1% NaCl, 500 IU nisin at HPP 55℃. This was followed by meat samples treated with 1% NaCl, 500 IU nisin, pH 4 at 55℃ and 1% NaCl, 100 IU nisin, HPP 55℃. Log reductions of 4.19 log cfu/ml, 3.66 log cfu/ml and 3.36 log cfu/ml were achieved in these three treatments compared to the control. The influence of nisin and pressure-thermal applications on spore inactivation were observed in these three treatments. The treatments revealed that higher concentrations of nisin (500IU compared to 100IU) and high pressure-thermal temperatures (HPP 55℃ compared to HPP 20℃) played a vital role in inactivating *B. cereus* NVH spores. Also, treatments with at least 100 IU nisin enhanced spore

inactivation with a relative increase in log reduction. For example, 1% NaCl, 100 IU nisin, HPP 55℃ showed a higher spore inactivation with an increased log reduction compared to 1% NaCl at HPP 55℃. Contrary to this, meat samples treated with 1% NaCl, 500 IU nisin, pH 4, HPP at 20℃ showed the least spore inactivation, also highlighting the importance of pressure-thermal temperatures on spore inactivation. Furthermore, experiment A also showed that pH 4 did not have much influence on inactivating the spores, as the highest spore inactivation was achieved without the pH 4.

Results from experiment B also showed that, the highest spore inactivation and log reduction were observed in meat samples treated with 1% NaCl, 500 IU nisin, pH 4 at HPP 55℃. This was contrary to what was observed in experiment A (1% NaCl, 500 IU nisin at HPP 55℃). However, there were similarities in the influence of nisin and pressure-thermal applications on spore inactivation and log reductions in the treatments; 1% NaCl, 500 IU nisin, pH 4 at HPP 55℃, 1% NaCl, 500 IU nisin at HPP 55℃ and 1% NaCl, 100 IU nisin, HPP 55℃ observed in experiment A and experiment B. In both experiments, these three treatments contained nisin with pressure-thermal treatments at 55℃. A comparison of these treatments with their respective controls and other treatments revealed that the presence of nisin and pressure-thermal treatments at 55℃ contributed to an increase in log reductions in both experiments.

Similarly, these treatments demonstrated that higher concentrations of nisin (500 IU compared to 100 IU) and high pressure-thermal temperatures (HPP 55℃ compared to HPP 20℃) played an important role in inactivating *B. cereus* NVH spores. At least 100 IU nisin enhanced spore inactivation with a relative increase in log reduction. For example, 1% NaCl, 100 IU nisin, HPP 55℃ showed a higher spore inactivation with an increased log reduction compared to 1% NaCl at HPP 55℃.

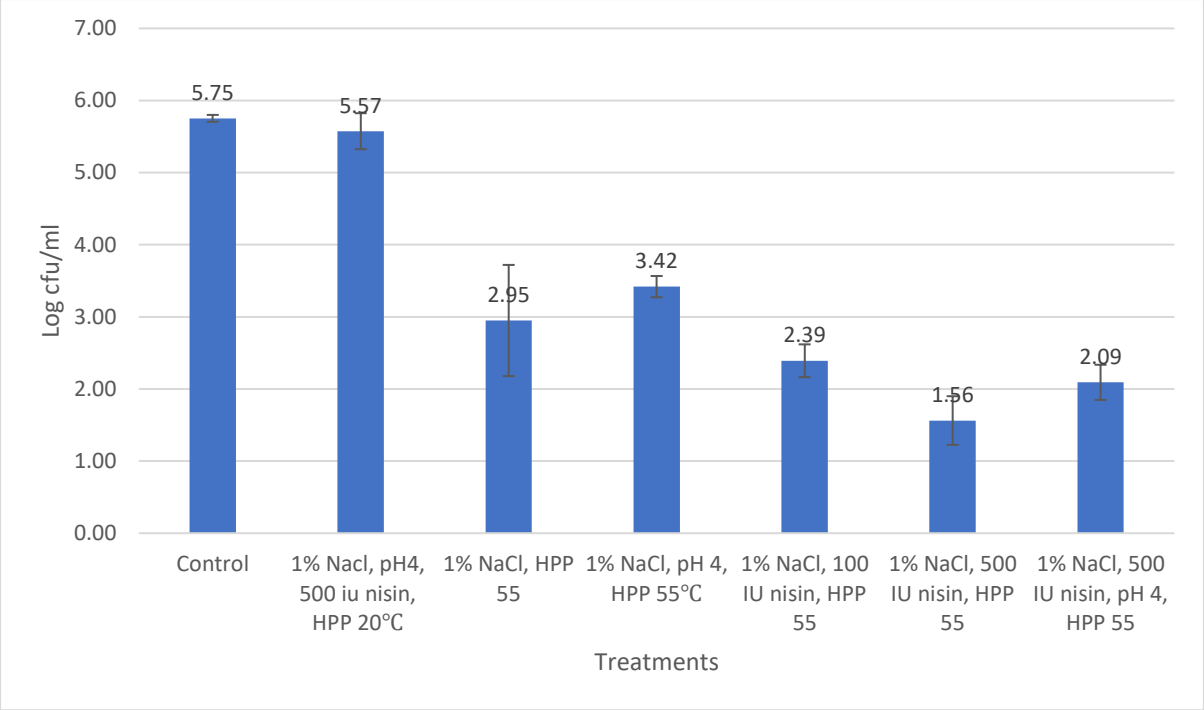


Figure 34: Experiment A: Inactivation of B. cereus spores in minced meat treated with a combination of preservatives and HPP processing at 600 MPa, 55℃.

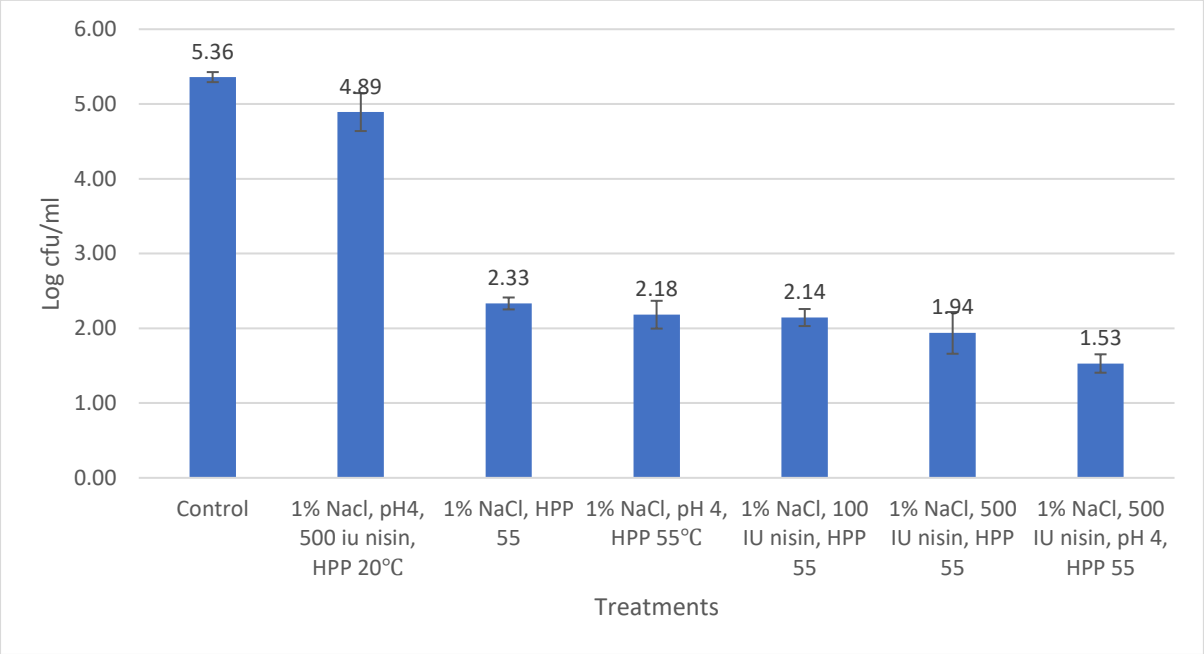


Figure 35: Experiment B: Inactivation of B. cereus spores in minced meat treated with a combination of preservatives and HPP processing at 600 MPa, 55℃.

Nisin in combination with non-thermal treatments, such as HPP, has resulted in a synergistic reduction in the population of various microorganisms, including bacterial spores (Aouadhi et al., 2013; Sobrino-López & Martín-Belloso, 2008). Experiments by Black et al. (2008).

demonstrated the germination and inactivation of spores of *Bacillus* species in buffer and milk subjected to high pressure (HP) and nisin. In this study, *Bacillus subtilis* and *Bacillus cereus* spores suspended in milk or buffer were treated at 100 or 500 MPa at 40°C with or without 500 IU of nisin. A second cycle of pressure at 500 MPa resulted in high levels of germination (4 log units) of B. subtilis spores in both milk and buffer; this was further increased to >6 logs units. Cycled HP reduced the viability of *B. subtilis* spores in milk and buffer by 2.5 logs, whereas the addition of nisin (500 IU ml) prior to HP treatment resulted in log reductions of 57 and 59% in phosphate buffered saline and milk, respectively. Also, treating four strains of *B. cereus* at 500 MPa for 5 minutes twice at 40°C in the presence of 500 IU ml1 nisin proved less effective at inactivating the spores of these isolates, with some strain to strain variability observed. Although combining HP and nisin resulted in high levels of germination of *Bacillus* spores, the aforementioned treatments did not result in complete inactivation (Black et al., 2008). Other studies also showed that the addition of nisin at 100 or 500 mg kg ^{1} suppressed total plate and anaerobic spore counts in processed cheese during 3 months of storage at 5 or 21 °C, and even the growth of *Bacillus stearothermophilus*, *Bacillus cereus*, and *Bacillus subtilis* was inhibited by 5 mg kg^{1} nisin (Wirjantoro et al., 2001). Relating this to our experiment, this study similarly illustrates that the addition of nisin (100 IU or 500 IU) resulted in an increase in log reductions with a corresponding increase in spore inactivation. Experiments by Periago and Moezelaar (2001) showed that nisin is more active at lower pH values, but the effect of temperature on its effectiveness is debatable. In this study, nisin was more active against *B. cereus* cells at lower pH values (6.30 and 5.75) than at pH 7.0 at 30°C, with different sensitivity of the strains tested. Comparing this to our study, a similar trend was observed in experiment B where meat samples treated with nisin (500 IU) at pH 4 showed a higher log reduction compared with the same treatment with nisin but no pH 4.

4.8 The combined effect of preservatives on *B. cereus* NVH vegetative cell growth in Bioscreen C.

The combined effect of preservatives and nisin on *B. cereus* NVH vegetative cell growth in Bioscreen C was investigated. This was a model experiment from the experiment described in *Experiment 4.7* to determine the effect of nisin and preservatives on vegetative cell growth. In this experiment, nisin at concentrations of 1000 IU and 200 IU were initially combined with 2% NaCl at pH 3 with bacterial suspension as described in the protocol. The vegetative cell growth of *B. cereus* NVH was determined using Bioscreen run at 37°C. Controls of stock 1 and stock 2 contained all preservatives and nisin without bacteria at final concentrations of 1%

NaCl, 500 IU/ml nisin, pH 4 and 1% NaCl, 100 IU/ml nisin, pH 4 respectively. Results of each treatment were performed in triplicate and the experiment was repeated twice.

(A)

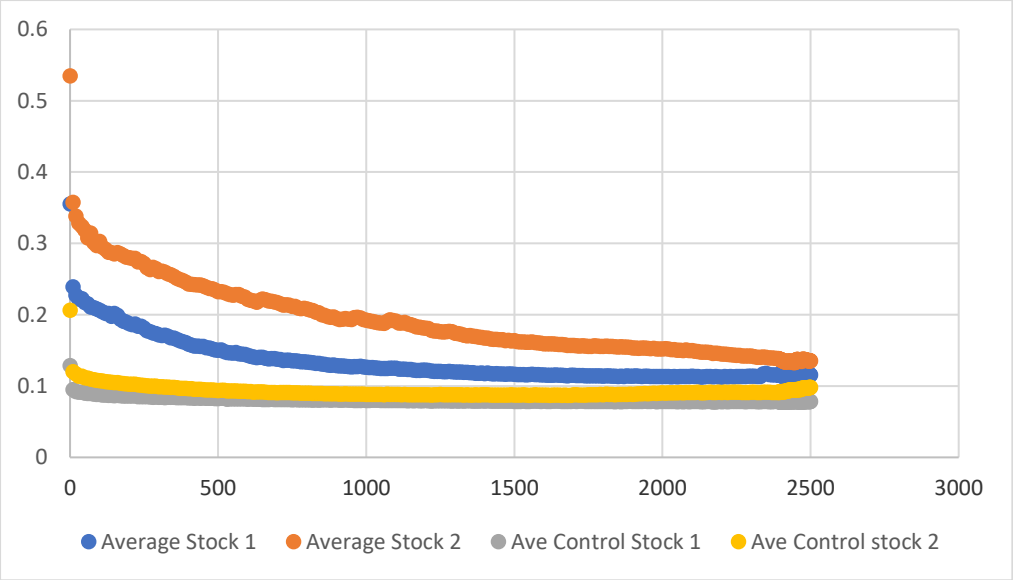


Figure 36: B. cereus NVH *vegetative cell growth when treated with a combination of 1% NaCl, 500 IU nisin, pH 4 with bacteria (Stock 1) and 1% NaCl, 100 IU nisin, pH 4 with bacteria (Stock 2) using Bioscreen at 37℃. Controls of stock 1 and stock 2 contained all preservatives and nisin without bacteria at final concentrations of 1% NaCl, 500 IU/ml nisin, pH 4 and 1% NaCl, 100 IU/ml nisin, pH 4 respectively.*

(B)

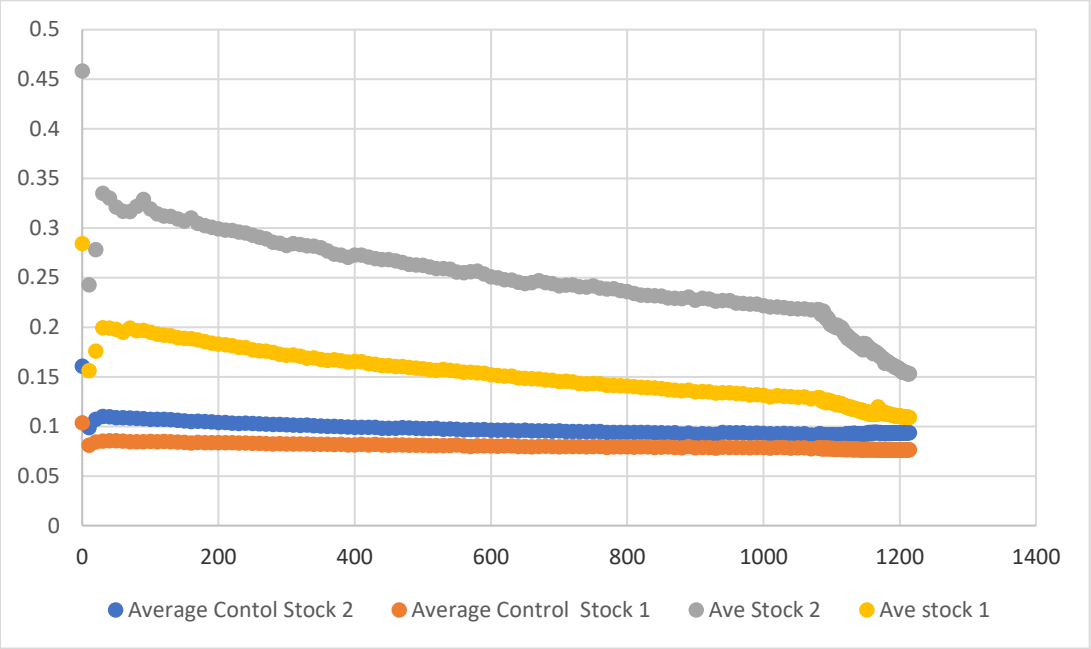


Figure 37: B. cereus NVH *vegetative cell growth when treated with a combination of 1% NaCl, 500 IU nisin, pH 4 with bacteria (Stock 1) and 1% NaCl, 100 IU nisin, pH 4 with bacteria (Stock 2) using Bioscreen at 37°C. Controls of stock 1 and stock 2 contained all preservatives and nisin without bacteria at final concentrations of 1% NaCl, 500 IU/ml nisin, pH 4 and 1% NaCl, 100 IU/ml nisin, pH 4 respectively.*

The results of both experiments showed that treatments with 1% NaCl, 500 IU nisin, pH 4 (Stock 1) had a lower OD (OD at 0.2) compared to treatments with 1% NaCl, 100 IU nisin, pH 4 (Stock 2) which has a higher OD (OD at 0.35) respectively. Even though different OD's were recorded at Stock 1 and Stock 2, it is difficult to define this as growth. Changes in OD could be influenced by changes in chemical reactions or colour changes in the medium. Also, the growth curves obtained from this experiment does not depict the usual sigmoidal growth for bacterial growth in Bioscreen. Nisin and salts were combined at pH 4 where no growth was to be expected. Therefore, it was difficult to attribute the effect on growth to pH or nisin. Higher pH values other than pH 4 could have better revealed the efficacy of nisin on *B. cereus* NVH growth.

5.0 Conclusion

The synergistic effect of pressure-thermal treatments in *B. cereus NVH* spore inactivation was revealed in this study.

- HPP alone could not achieve the highest spore inactivation in minced meat. However, a synergy of pressure-thermal (HPP-55℃) treatments resulted in an increase in spore reduction (3 log reductions) in the meat matrix. Although high temperature/time profiles contributed to an increase in spore inactivation, the effect of matrix composition on spore thermal resistance is vital. An increase in spore inactivation was recorded in LB (log 4.1 cfu/ml at 97℃ for 10 mins) compared to the minced meat (log 3.35 cfu/ml at 97℃ for 10 mins), illustrating the shielding effect of food matrices on spore thermal resistance.
- The inclusion of more hurdles such as combined preservatives (NaCl, KCl, nisin) at higher concentrations and low pH (pH 4) resulted in bacterial stress which increased spore inactivation and growth inhibition in *B. cereus NVH.*
- Even though, there was no growth at pH 4, the combination with other hurdles such as increased refrigeration temperatures of 15℃ at prolonged times, lower salt concentrations could initiate bacterial growth. On the contrary, $NaNO_2$ in varying concentrations had no effect on the growth of *B. cereus NVH* spores. The ability of *B. cereus NVH* vegetative cells to grow and multiply at 8℃ with time, makes it a huge food safety hazard. An effective way to control *B. cereus* growth measure is to ensure that the rate and extent of cooling is <4℃. This should be rapid to prevent potential spore germination and growth of vegetative cells.

5.1 Future Works

The application of pressure-thermal treatments (PATS) and preservatives in *B. cereus* NVH spore inactivation has shown promising outcomes which require further investigations. Areas in this project that require further studies include:

- Investigate the use of PATS on *B. cereus* NVH in other food matrices like fish and foods with mild preservation.
- Increase temperature/time profiles (higher than 55℃) in the use of PATS to determine the extent bacterial spore inactivation.
- Explore the combination of other preservatives in *B. cereus* NVH spore inactivation.

- The combination of nisin, salts (NaCl), pH 4 and HPP in *B. cereus* NVH spore inactivation was promising and should be investigated further. The combination of nisin with other salts like KCl (for health purposes), HPP at pH levels other than pH 4 should be tested to reveal the efficiency of the other preservatives.
- Investigate the potential of herbal extracts and essential oils in *B. cereus* NVH spore inactivation and vegetative growth inhibition as a more sustainable alternative.
- *B. cereus* NVH is psychrotrophic and can grow at 8°C with time. The ability of this strain to grow at temperatures below 8°C should be investigated. This could be studied with a combination of preservatives, in food matrices with prolonged time intervals to prevent potential food safety hazards during chilled storage and enhance the shelf-life of foods.

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Appendix

Calculation for nisin/IU for HPP experiment BioScreen

1 1g Raw nisin in 1ml 002 M HCl -> 1000 mg/ml = 1 000 000 ug/ml. (we used 0,5 ml + 4,5 ml dH2O, but it will be same dilution factor)

2 Diluted with 9 ml HCl -> 1 000 000 ug/10 ml=100 000 ug/ml

3 2,5 % is active Nisin (AN) -> 100 000 ug x 2.5% =2500 ug (AN)/ml=2,5mg/ml

4 This is (from 3) the **stock**-> 2,5mg AN/ml = 2 500 ug/ml

Since 1ug AN= 40 IU -> This gives 100 000 IU/ml=100 IU/ul (this is the same as Lee et al)

Stock solution used in HPP experiment.

5 250 ug (25% of 1 ml of stock solution, no 4) added to 6 g meat -> 625 ug/6g=104,16ug/g Since 1ug AN= 40 IU -> 104,16 x 40 IU/ug=4166 IU/g in the meat

6 1225ug (of stock 4) added to 6 g meat-> 3125ug/6g=520,8 ug/g

Since 1ug AN= 40 IU -> 520,8 x 40 IU/g= 20800 IU/g

BioScreen experiment

7 If the stock is used directly from no. 4, then the wells and calculations will be as in table 1. The volumes of 1 and 10 is examples showing the final concentrations in the wells.

Table 1

Stock (4)	LB/NaCl/pH	ul in well	IU/well	IU/ul	IU/ml(g)	
1ul	199	200 ul	100 IU/200	500 IU/1000 ul	500 IU/ml	
10	190	200ul	500 IU/200	2500 IU/1000 ul	2500 IU/ml	

8 We wanted concentrations of 100 and 500 IU, as in the work of Lee et al.

If stock solution (4) is diluted 10 X, this gives 0,25mg AN/ml = 2 50 ug/ml = 10 000 IU/ml = 10 IU/ul

Then it is possible to add 50:50% and obtain 500 IU/ml. Five times more diluted stock (4) will give 100 IU/ml.

Table 2

Stock (4)	LB/NaCl/pH	ul in well	IU/well	IU/ul	IU/ml(g)	
100 ul	100 Double concentr.	200 ul	100 IU/200ul =0,5 IU/ul	500 IU/1000 ul	500 IU/ml	

Proposed Protocol

If to obtain 500 IU/ml, stock solution (4) is diluted 10 X, this gives 0,25mg AN/ml = 2 50 ug/ml = 10 000 IU/ml = 10 IU/ul

Then to obtain 1000 IU/ml, stock solution (4) is diluted 5X, this gives 0.5 mg AN/ml = 500 ug/ml = 20 000 IU/ml = 20 IU/ul

If to obtain 100 IU/ml, stock solution (4) is diluted 15X, this gives 0,167mg AN/ml = 166.7 ug/ml = 6667 IU/ml = 6.7 IU/ul

Then to obtain 200 IU/ml, stock solution (4) is diluted 8X, this gives 0,3125 mg AN/ml = 312.5 ug/ml = 12500 IU/ml = 12.5 IU/ul

1. Make stock solution of 2% NaCl solution by dissolving 1.689 g NaCl in 20 ml LB medium (20ml of 2% NaCl)
2. Prepare respective dilutions of nisin solution using the 2% NaCl (instead of dH20)

   Stock 1: 1 ml of stock nisin in 4 ml of (20 ml 2% NaCl) = 1000 IU/ml=20 IU/ul (x5)

   Stock 2: 1 ml of stock nisin in 7 ml of (20 ml 2% NaCl) = 200 IU/ml=12.5 IU/ul (x8)
3. Adjust solutions to pH3
4. Set in wells as follows

Sample A

500 IU/ml = 50:50 (100 ul bacteria: 100 ul Stock 1)

Sample B

100 IU/ml = 50:50 (100 ul bacteria: 100 ul Stock 2)

Control 1 = 1000 IU/ml = Stock 1

Control 2=200 IU/ml = Stock 2

1	2	3	4	5	6	7	8	9	10	11	control
200 ul of Sample A	-	-	-	-	-	-	-	-	-	-	Control 1
	-										-
	-										-
200 ul of sample B	-										Control 2
	-										
	-										

EFSA journal on nisin

According to EFSA ANS Panel (2006a, b), nisin (nisin A) is a polypeptide composed of 34 amino acids and belongs to class I bacteriocins. Bacteriocins are proteins/peptides naturally produced by bacteria to inhibit the growth of other bacteria which is produced via fermentation by Lactococcus lactis subsp. lacti.

The Panel noted that the applicant provided analytical results of five independently manufactured batches of Nisaplin® (according to the applicant Nisaplin® is a commercial preparation of nisin A, containing 1,000 IU/mg) to demonstrate the compliance with nisin (E 234) specifications ('Documentation provided to EFSA' No. 1). The Panel noted that 1 IU would correspond to 0.025 ug of nisin and therefore 1 ug of nisin is equivalent to 40 IU (JECFA, 2013). Based on the information provided by the applicant, the Panel considered that the Nisaplin® is consistently manufactured within the defined specifications for Nisin (E 234). The high-performance liquid chromatographic (HPLC) method used for the assay is validated against the resazurin assay. The resazurin assay is performed as described in the JECFA monograph for nisin (JECFA, 1969, 2013).

Preparation of nisin stock solution by Lee et al (2015)

Preparation of nisin and addition to beef jerky Nisin (Sigma-Aldrich, USA) was used as a form of stock solution. A standard stock solution of nisin containing 1×10^5 IU/mL was prepared, by dissolving 100 mg of nisin in 0.02 M HCl (1 mL) and adding 9 mL of distilled water. Nisin was added at concentrations of 100 IU/g and 500 IU/g, respectively to the beef jerky